THE
BIOLOGICAL BULLETIN

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It is with deep regret that the Biological Bulletin
records the death, on January 4, 1943, of
Dr. Gary Nathan Calkins, a member of
the Editorial Board since 1927.

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TRANSPLANTATION AND INDIVIDUALITY DIFFERENTIALS IN INBRED STRAINS OF RATS

LEO LOEB, H. D. KING, AND H. T. BLUMENTHAL

(From the Laboratory of Research Pathology, Oscar Johnson Institute, Washington University School of Medicine, St. Louis, and from the Wistar Institute, Philadelphia)*

In two previous papers (1927, 1931), Loeb and King have reported on the individuality differentials of rats inbred in the Wistar Institute for a considerable number of years by Dr. Helen Dean King. In this paper we shall report on a third series of experiments, carried out during the last two or three years, in which rats from inbred generations 102 to 106 were used. There was added, also, a small series of experiments with rats from inbred generations 91 and 92. However, in order to evaluate the changes which have taken place in the course of the continued inbreeding it will be necessary to give also the main data obtained in the first and second series of transplantations made with the earlier inbred generations. A comparison can be readily made if instead of discussing isolated data, we group experiments of a similar kind together by adopting a system of grades and determining the average grades obtained in each case. We have already adopted, in previous papers, such a system of approximately quantitative grading of the findings, and we have discussed these grades more fully on former occasions. Here, the principles underlying the choice of these grades will be only briefly explained. To simplify this task, we shall consider mainly the results obtained in transplantation of thyroid, cartilage, fat tissue, and also of the other organs often associated with these, namely, parathyroid, bone, bone marrow, striated muscle. It will not be necessary at this time to enter into a discussion of the corresponding reactions shown in all types of tissues used in our grafting experiments. The grades used range between 1 and 3+ (3.25); 3+ and 3 are the grades characteristic of autogenous transplantations. In this latter, the tissue—in particular, thyroid gland—is well preserved, and while at first there may be some irregularities in the structure of the graft, it gradually assumes the structure of the normal organ. Marked lymphocytic infiltration is lacking, but at early periods some very small collections of lymphocytes may be

* The work from the Laboratory of Research Pathology of Washington University School of Medicine was carried out previous to June 1, 1941, when this laboratory was discontinued. The experiments mentioned in this paper were done with the aid of a grant from The International Cancer Research Foundation.

seen; subsequently, these usually disappear. Likewise, the connective tissue ingrowth is restricted and an invasion of the fat tissue by small vacuolated cells and by fibrous tissue is absent. Grades 3− (2.75) and 2+ (2.25) are given if the tissues are, on the whole, well preserved but if a reaction is definitely noticeable. Various degrees of lymphocytic infiltration and a somewhat increased activity of the connective tissue may appear and cause a limited injury to the transplant. Such reactions may be seen when donor and host are related. If these reactions are more marked and lead to a partial destruction of the transplant, the grade 2 is given; this indicates a somewhat greater strangeness of the -indi viduality differentials. In typical, more severe homoio-reactions the grades range between 2− (1.75) and 1. Grade 2− is given if the thyroid gland is severely invaded by fibrous tissue and a considerable part of the acinar tissue has been destroyed; but at least one-half of the organ has escaped destruction at the time of examination, which in most cases is between 20 and 30 days following transplantation. There is, in these cases, a definite lymphocytic infiltration, provided injury to the tissue has not led to a marked diminution in the effectiveness of the individuality differential substances. Grade 1+ (1.25) signifies the survival of only a small part of the thyroid gland. The reaction in the fat tissue is very severe. Grade 1 is applied to results in experiments in rats and guinea pigs in which the thyroid has been entirely destroyed and the fat tissue is largely replaced by fibrous tissue.

The changes in other organs or tissues, on the whole, correspond to those taking place in thyroid and fat tissue and this correspondence makes possible the grading of the results obtained in all tissues from the same donor and transplanted into the same host. For instance, if ovaries are transplanted together with thyroid, cartilage and fat tissue, we find corresponding intensities of reaction and injuries of the various constituent structures of this organ. In the most favorable cases, large follicles, and even corpora lutea, are found. In somewhat less favorable cases only medium-sized or small follicles develop; a still more unfavorable reaction is indicated if merely primordial follicles survive, without undergoing further growth processes. If the reaction is still more severe, no follicles are seen, but merely a cyst and ducts of the germinal epithelium, medullary ducts, spindle-cell connective tissue, and interstitial gland tissue, together with necrotic remnants of the transplanted ovary. At last, only some interstitial gland may be found, or even this tissue may be lacking and necrotic material with fibrous tissue may be all that is left. However, under all circumstances it is necessary to make allowances for the occurrence of accidental injuries to the transplant. This can be recognized in many instances, but even if it should be difficult, errors in the appraisal of the reaction can be avoided by making a series of experiments instead of a single one and then relying on an evaluation of the total results. While this method of grading can claim only approximate exactness, still it is very helpful in comparing the results in the interaction of different types of individuality differentials and in making possible a concise and sufficiently accurate expression of a large number of otherwise separate data.

The principal results obtained in the first series of experiments (1927) are as follows:

SERIES I

Inbred Families A and B

In subseries 1, generations 37 and 38, 40 and 41, in subseries 2, generations 46 and 47 were used.

Subseries 1, family A (different litters): grade 1.96 (20 rats)
Subseries 2, family A (" "): " 1.12 (4 rats)
Total family A (" "): " 1.82 (24 rats)

Subseries 1, family B (different litters): grade 2.1 (22 rats)
Subseries 2, family B (" "): " 1.55 (5 rats)
Total family B (" "): " 1.92 (27 rats)

Families A and B combined: grade 1.87 (51 rats)

Subseries 1, family A to family B: grade 1.74 (11 rats)
Subseries 2, family A to family B: " 1.57 (7 rats)
Total family A to family B: " 1.67 (18 rats)

Subseries 1, family B to family A: " 1.61 (16 rats)
Subseries 2, family B to family A: " 1.33 (3 rats)
Total family B to family A: " 1.57 (19 rats)

Total inter-family A and B transplantations: Combined grade 1.62 (37 rats)

Exchange of tissues between members of the same litters (brothers and sisters) in families A and B

Subseries 1, family B: grade 2.87 (17 rats) (40th and 41st generations)
Subseries 2, family A: grade 2 (6 rats) (42nd generation)
Total families A and B (40–42 generations): grade 2.5 (23 rats)

Subseries 3, family A: grade 1.37 (6 rats) (46th and 47th generations)
Subseries 4, family B: grade 1.78 (7 rats)
Total families A and B (46–47 generations): grade 1.59 (13 rats)

Total families A and B (members of same litters): grade 2.26 (36 rats)
2 subseries of family A: grade 1.68 (12 rats)
2 subseries of family B: " 2.55 (24 rats)

These data are summarized in Table I.

In evaluating these grades, we must attach greater importance to those experiments in which the figures represent the averages of a large number of rats than to those in which the figures are based on a relatively small number; in the latter, variable factors of an accidental nature may preponderate. This is a point to be considered if there is some discrepancy between the figures in related series of experiments. However, on the whole the figures here agree very well with each other.

The transfer of tissues from members of family A to members of family B, and vice versa, should correspond about to ordinary homoiogenous transplantations. The average grade is, here, 1.62, which is perhaps very slightly better

TABLE I (Series I)

Donor and host	Grades	Combined grades
Family A to family A (different litters)	1.82 (24 rats)	1.87 (51 rats)
Family B to family B (different litters)	1.92 (27 rats)	
Family A to family A (litter mates)	1.68 (12 rats)	2.26 (36 rats)
Family B to family B (litter mates)	2.55 (24 rats)	
Family A to family B	1.67 (18 rats)	1.62 (37 rats)
Family B to family A	1.57 (19 rats)	

than might be expected. This grade may be compared with that of transplantations between members of family A: 1.82, and between members of family B: 1.92, or an average grade of both kinds of intra-family transplantations: 1.87. There is a slight improvement, a slight diminution in the severity of the reactions in these intra-family transplantations as compared with the inter-family transplantations. Still somewhat higher is the average grade of transplantations between litter mates (brothers and sisters) in family A as well as in family B. Here the total average grade is 2.26 (in the larger series of experiments in family B the average grade is 2.55 and in the smaller series in family A it is 1.68). We may then conclude that as a result of close inbreeding for 37 to 47 generations in families A and B, only a very slight progress towards a homozygous condition has been accomplished.

Series II. In this series of transplantations, rats inbred for 60 to 67 generations were used. The following is a summary of the principal results in this series.

If we compare the grades in the first and second series, we find that transplantations between different litters in family A, and again in family B, show no improvement in series II over the corresponding grades in series I; on the contrary, the average grades in series II happen to be somewhat lower. This may perhaps have been due to a smaller number of brother-sister matings which different litters in series II had in common. On the other hand, in the latter series the grades between litter mates in family A, as well as in family B, are not only better than the grades obtained in transplantations between different litters, but they are also better than the transplantations between litter mates in series I. If the results of transplantations between brothers and sisters are compared in inbred rats, the variable factor of the branching-off of certain lines at different levels of brother-sister matings is eliminated and the improvement in series II in this type of grafts may thus indicate a certain progress towards a homozygous condition, although this cannot be maintained with certainty. Exchange of tissues between families A and B (inter-family transplantations)

TABLE II (Series II)

Donor and host	Grades	Combined grades
Family A to family A (different litters)	1.16 (16 rats)	1.49 (49 rats)
Family B to family B (different litters)	1.65 (33 rats)	
Family A to family A (litter mates)	2.60 (17 rats)	2.71 (36 rats)
Family B to family B (litter mates)	2.81 (19 rats)	
Family A to family B, or family B to family A	1.37 (32 rats)	
Homoiotransplantation in non-inbred families	1.36	
Hybrids $(A\times B)F_4$ (or F_5) to hybrids $(A\times B)F_4$ (different litters)	1.29 (12 rats)	
Hybrids $(A\times B)F_4$ (or F_5) to hybrids $(A\times B)F_4$ (litter mates)	1.80 (26 rats)	
Family A or family B to hybrids $(A\times B)F_4$	1.50 (13 rats)	
Hybrids $(A\times B)F_4$ to family A or family B	1.39 (13 rats)	

corresponds to a severe homoio-reaction, as might be expected. Similar are the grades of transplantations between hybrids $(A\times B)F_4$. These grades are improved to a certain degree if hybrids which are litter mates are used. But in these transplantations the results are not so good as in brother-to-brother transplantations in family A or in family B. Such a difference might be expected, because in hybrids there is a greater chance for unlike genes to accumulate in brothers when both A and B may contribute to the genes of the fertilized germ cells.

Transplantations from parent to hybrid give somewhat better results than the reciprocal transplantations, but both elicit severe homoiogenous reactions as an indication that a homozygous genetic constitution has not yet been reached in either family A or family B. It is of interest to note that in both series I and II the grades in family B are higher than in family A. On the whole, then, while it is possible that a further slight progress towards a homozygous condition in family A and in family B has been made in continued propagation by brother-sister matings in the interval between the 37th to 47th generations and the 60th to 67th generations, this progress, if present at all, is not very marked.

Series III. This is the series of experiments which has been carried out during the last few years and it will be discussed, therefore, in somewhat more detail. A small number of experiments were made with rats of the 91st and 92nd generations. These were young animals and at the end of the experiments, weighed between 125 and 140 grams. Transplantations into three litter mates, serving as hosts, gave the grade 2.95; very closely approaching the results obtained in autogenous transplantations. In seven transplantations from family B to family A the grade was 1.48, if the examination took place after 20 days, and it was 1.84 in 7 rats after 12 days. The grade after 20 days was very similar to the grades obtained in the corresponding transplantations in series I and II. No essential changes have taken place in this type of grafting between two different families in the course of long-continued brother-sister matings. The grades for transplantations between litter mates are somewhat better than in the corresponding ones in series II, and they are distinctly better than in series I, indicating further progress to a homozygous condition after 91 to 92 continuous brother-sister matings. However, in evaluating the grades obtained in transplantations between litter mates, the small number of experiments, of which this figure represents the average, has to be considered.

In generations intermediate between the 92nd and 104th generations, family B died out and from then on only family A and hybrids $(A\times B)F_4$ or $(B\times A)F_4$ were available for experiments. A number of transplantations were carried out with generations 104 to 106 of family A and with the hybrids. Younger rats, one month and one or two weeks of age, or somewhat older ones, four and a half to five and a half months of age and weighing between 185 and 200 grams at the beginning of the experiments were used for this purpose. Examination as a rule took place after 20 days. Usually three pieces, namely, thyroid, cartilage with fat tissue, and striated muscle tissue were transplanted, and in some experiments the transplants from one kind of donor were transferred to the right side, and those from another kind of donor were transplanted to the other side of the same host.

Group A. Transplantations of organs in young rats

(*a*) Transplantations between members of different litters in family A: average grades, 2.81 and 2.83; (*b*) between litter mates: average grades, 3.15; 3.15; 3.12 and 3.07; (*c*) from hybrids F_4 between families A and B to family A: average grades, 3.12 and 3.10; (*d*) between litter mates of hybrids $(A\times B)F_4$ or $(B\times A)F_4$: average grade, 3.12—the same grade was found between hybrids which belonged to different litters. There is no difference, or only a very slight one, in the reactions obtained in these various transplantations, the grades between non-litter mates being somewhat lower than those between litter mates; but the significance of this difference is not great, because only four transplantations were carried out between members of different litters. On the other hand, even the grades for transplantations from hybrids F_4 to family A, which might have been expected to lead to more severe reactions, were as good as those between litter mates of strain A. We must therefore assume that the young age of these rats helped to diminish the severity of the reactions, which was also diminished by the long-continued inbreeding, causing an approximately autogenous condition of the individuality differentials in family A and in those

members of family B which were components of hybrids $(A \times B)F_4$. However, in one experiment the transplantations from hybrids F_4 to family A, in accordance with expectations, gave a somewhat more severe reaction; the average grade was here slightly below 2. In this case, the host reacted definitely against two transplants which contained strange B genes; the threshold which determines an antagonistic reaction had evidently been passed.

Group B. Transplantations in somewhat older rats

These rats varied in age between four and a half and seven and a half months and belonged to the 102nd, 103rd, or 104th generation. The kind of transplantations were about the same as in the younger animals. Transplantations were made (*a*) between litter mates of family A: grade 3.04 in rats aged $4\frac{1}{2}$ to $5\frac{1}{2}$ months, and grade 2.56 in rats aged $6\frac{1}{2}$ to $7\frac{1}{2}$ months; (*b*) between different litters of family A: grade 2.93 (age of rats $4\frac{1}{2}$ to $5\frac{1}{2}$ months), and grade 2.4 (age $6\frac{1}{2}$ to 7 months). The grades between litter mates are only very slightly better than those between members of different litters in family A. These grades are not as good as those in the younger rats, and also among the older rats the severity of the reaction increased somewhat with increasing age; transplantations were also made (*c*) between hybrids $(B \times A)F_4$ and hybrids $(A \times B)F_4$: grade 2.18; (*d*) from family A to hybrids $(A \times B)F_4$: grade 2.39; (*e*) from hybrids $(A \times B)F_4$ or $(B \times A)F_4$ to family A: grade 2.23. The grades in transplantations between hybrids themselves and between hybrids and members of family A, in accordance with expectations, are less good than those between members of family A. These differences come out in the older rats, while they are not present in the corresponding transplantations in younger rats. However, also in the older rats there are no very sharp differences between these various groups.

We shall now give brief abstracts of our microscopic findings in some of these transplantations, which may serve as examples. (1) Transplantations in young rats, family A, inbred for 104 or 105 generations. These rats were 37 to 44 days old at the time of transplantation; examination took place 20 days after transplantation. Pieces from litter mates were transplanted to the right side and pieces from different litters to the left side of the host. There was no definite difference between pieces from the two sides. The transplant of striated muscle tissue was well preserved. There were well-preserved, thin muscle fibers, either close together or separated by some hyaline tissue. Some thick fibers were surrounded by hyaline tissue and in certain instances also by strands of fibroblasts. Necrotic muscle fibers were, in places, surrounded by foreign-body giant cells. There were well-developed nuclear chains, but there was less tendency to the formation of nuclear masses, less crowding, and better cross-striation of muscle fibers in the rat than in the mouse. On the whole, the multiplication of nuclei in fibers was moderate. On the average, the least proliferation of muscle nuclei was found where the thickness of the muscle fibers was greatest and where the cross-striation was best. Where the muscle fibers were very thin and much drawn-out, they often resembled fibroblasts. Some muscle fibers might be changed into a light material, which did not stain with eosin.

The thyroid transplants were well preserved. There was a ring of two to three layers of acini of medium or small size. The acinar epithelium was flat cuboidal, or, occasionally, cuboidal in shape; the center consisted of fibrillar-

cellular connective tissue, in which there were some lymph and blood vessels. There were also some ducts lined with squamous epithelium, or there were some epithelial pearls in the center of the transplant, and here, too, fat cells were found, indicating a favorable result of the transplantation.

Cartilage and fat tissue were well preserved. The strands of fibrous tissue observed in the fat tissue presumably were caused by the injury sustained during operation. Otherwise there was no connective tissue ingrowth, no infiltration with lymphocytes, and no accumulation of small vacuolated cells between the fat cells. Necrosis occurred in a small piece of cartilage—probably as the result also of injury at the time of transplantation—and surrounding the latter was a plate of regenerated perichondral cartilage; but in the piece from the left side of the host, some necrotic cartilage, without regenerated cartilage plate, was seen.

As to the behavior of the lymphocytes in the muscle tissue from the right side (litter mates), there were merely a few lymph vessels filled with lymphocytes. In the piece from the left side (non-litter mates) there were some similar lymph vessels, and, in addition, some diffuse lymphocytic infiltration. In the thyroid transplant from the right side the lymphocytic infiltration was very moderate; there were a few lymph vessels filled with lymphocytes, and at the two poles, small collections of lymphocytes were seen. In the thyroid transplant from the left side a dense lymphocytic infiltration was noted in the center of the gland around squamous cell nests. In places, lymphocytes penetrated between acini and separated them. Lymph vessels were stuffed with lymphocytes. Also, the parathyroid showed lymphocytic infiltration. In the cartilage-fat tissue transplants of the right side (litter mates) there were no lymphocytes. On the left side (non-litter mates) there was some lymphocytic infiltration in the fat tissue along the living cartilage. This experiment exemplifies well the correspondence between multiple transplants from the same donor into the same host.

(2) Transplantation in young rats from $(B \times A)F_4$ to A (right side), and from $(A \times B)F_4$ to A (left side). In the muscle transplants on the right and left sides there was some lymphocytic infiltration in various places between groups of muscle fibers. On the right side the muscle was less well preserved, more necrosis and more lymphocytic infiltration had taken place, and many nuclear masses were seen. Around some cloudy muscle fibers, foreign-body giant cells had formed. In both thyroid transplants there was intense lymphocytic infiltration; the ring of acini was incomplete; some groups of acini were separated by lymphocytic masses. Lymphocytes penetrated also into the colloid, where they disintegrated. The masses of lymphocytes had destroyed a number of acini. In the cartilage-fat tissue, cartilage was well preserved. On the left side there was more fat tissue replaced by fibrous tissue than on the right side. In both transplants there was some lymphocytic infiltration along the perichondrium and, in some places, also between the fat cells. In these experiments the reaction is more severe than in transplants from A to A.

(3) In the somewhat older rats, the grades in some of the transplantations within the inbred family A approached those of autogenous reactions. There were occasionally slight lymphocytic infiltrations in the thyroid, in which there were several layers of acini, but there were no lymphocytes between the acini. The muscle transplant was well preserved, with much cross-striation and with nuclear chains and masses. The fibers with medium thickness were best pre-

served as a rule. Some muscle fibers became hyaline and the preserved fibers were usually embedded in hyaline tissue. There were occasionally some small collections of lymphocytes in the transplant. In the cartilage-fat-tissue transplant some thickened septa with a small number of lymphocytes were observed.

(4) $(B \times A)F_4$ to $(A \times B)F_4$ (older rats—experiment 8). The muscle transplant showed very intense lymphocytic infiltration throughout, and this destroyed the fibers. Certain muscle fibers disintegrated, others became hyaline. There were some nuclear chains. The thyroid ring was incomplete. Single acini might be separated by connective tissue and lymphocytes. Much lymphocytic infiltration had taken place and lymph vessels might be stuffed with lymphocytes. The lymphocytes penetrated between the acinar cells into the colloid. The greater part of the thyroid had been destroyed. In the cartilage-fat-tissue transplant some cartilage had become necrotic and dense fibrous tissue surrounded it. Much fat tissue was destroyed, but there were still some groups of fat cells. In some places more fat tissue was preserved, with fibrous strands and definite lymphocytic infiltration in the hyaline connective tissue. Lymph vessels were stuffed with lymphocytes in the connective tissue and fat tissue.

(5) Experiment 61b. 91st and 92nd generations, family B to family A; (20 days). In the thyroid the results varied between those in which no thyroid was left, to those in which the remnant of the transplant consisted mainly of compressed acini, with much lymphocytic infiltration and some connective tissue ingrowth. In the cartilage-fat-tissue transplant, much fat tissue, and in some places almost all of it, was replaced by fibrous tissue. If much lymphocytic infiltration occurred in the thyroid, usually a certain amount was found also in the fat tissue. Bone marrow was not preserved, but as a rule some transplanted muscle fibers were preserved. Some lymphocytic infiltration had taken place in this transplant.

The principal results obtained in series III are summarized in Table III.

Table III shows that the grades in the younger group of rats are consistently higher than in the somewhat older rats; this consistency in the difference between the grades in these two groups makes it very probable that this difference is significant, even in cases in which the averages are based on a small number of individuals. The differences are, however, not very great in transplantations within the inbred family A; they are greater if we compare the transplantations between hybrids and from hybrids to parent-family A in the old and young groups. The grades in transplantations from family B to family A, both belonging to the 91st and 92nd generations, are very low; as was to be expected, they correspond to homoiogenous reactions.

If we compare the grades obtained in series III with those of series II, we notice that they are much better in the former. In transplantations within family A, the grades have advanced from 1.16 in series II to about 2.75 in series III. Among litter mates within family A they have advanced from 2.60 in series II to about 2.90 in series III. This improvement is not so great as in the transplantations between non-litter mates, which is due to the fact that there was already a considerable improvement among the grafts between litter mates in series II, as compared to those in series I with a corresponding grade of 1.68. There has been also a great improvement in the grade of transplantations between hybrids F_4 (non-litter mates). The corresponding grades are 1.29 in the II

TABLE III (Series III)

Donor and host	Grades	Grades
A to A (different litters)	2.82 (7 rats)	2.72 (10 rats)
A to A (litter mates)	3.10 (22 rats, including 3 from the 91st and 92nd generations)	2.77 (7 rats)
$(A \times B)F_4$ and $(B \times A)F_4$ (different litters as hosts and donors in various combinations)	3.12 (6 rats)	2.18 (2 rats)
$(A \times B)F_4$ to $(A \times B)F_4$ (litter mates) $(B \times A)F_4$ to $(B \times A)F_4$	3.12 (6 rats)	
A to $(A \times B)F_4$		2.39 (4 rats)
$(A \times B)F_4$ or $(B \times A)F_4$ to A	2.87 (10 rats)	2.23 (4 rats)
B to A (91–92 generations)	1.48 (7 rats) 20 days 1.84 (7 rats) 12 days	

series and about 2.50 in series III. Transplantations from hybrids to family A (or B) show a considerable improvement from grade 1.39 in series II to about grade 2.50 in series III; and between litter mates of hybrids there is a change from 1.80 (series II) to 3.12 (young rats) in series III. In transplantations from family A to hybrids the advance is marked, in series II the grade being 1.50, in series III, 2.39 (older rats). Where in series III there were grades available in both groups, namely, in those of old and of young rats, a somewhat arbitrary intermediate grade was chosen, but one nearer to the grade of the older rats. In contrast to the change which has taken place in the interaction between the individuality differentials of the members of inbred strains, in the course of long-continued brother-sister matings, no essential change has occurred if the individuality differentials interacted between members of family A and of family B. In this case, the grade (1.48) obtained in series III, when the examination took place after 20 days, indicates a typical homoiogenous reaction. As was to be expected, the reaction was not yet fully developed in its full strength and the grade therefore was somewhat higher (1.84) if the examination took place after 12 days.

DISCUSSION

A comparison of the reactions observed in these three series of transplantations shows that a continuous progress to a homozygous condition has been made. In the first series, there was only a slight indication of an improvement in grades over the grades in ordinary homoiogenous and syngenesious transplantations. A further slight progress was noted in the II series, but the greatest advance was made in the interval between the II and III series. The first series comprised

the 37th to 41st generations; the second series, the 60th to 67th generations; and the III series, the 91st and 92nd generations as well as the 102nd to 107th generations. After about forty generations, there was then only a very slight progress towards an autogenous character of the individuality differentials; some advance was made after 60 to 67 consecutive brother-sister transplantations, and still more in the 102nd generation; but even at that time, no completely homozygous condition had been attained. This finding is indicated especially by the transplantations into which the hybrids entered; but it is noticeable also in the transplantations within the inbred family A. Of interest in the third series is also the difference in the grades in the group of the young and the somewhat older rats, which agrees with the general observation that when donors and hosts are very young, the reactions are milder than in older animals. This difference cannot be due to a lack of individuality differentials in the former, because they are present; but it is due, rather, to a lesser sensitiveness to strange individuality differentials or to a not yet fully developed mode of reaction in the younger animals. In addition, the fact must be taken into account that younger tissues grow more vigorously than older ones, and this condition is associated with a greater ability to overcome the effect of the antagonistic reactions of the host; it may also be that tissues growing more rapidly do not give off individuality differential substances in as large amounts as the more differentiated tissues, which metabolize in carrying on their function. In accordance with these considerations, we noticed that in the group of younger rats the grades are higher even in transplantations from hybrids to an inbred parent strain, where the derivatives of strange genes are introduced into the host. Another important conclusion to be drawn from these experiments is that although so large a number of consecutive brother-sister transplantations were carried out, a completely homozygous condition has not yet been reached in these families, as is shown by the results of transplantations within the inbred family. This conclusion agrees with the findings obtained also in transplantations in inbred families of guinea pigs and mice. In none of these, even after years of consecutive brother-sister matings, has a completely autogenous state of the individuality differentials been attained. This effect, which is contrary to expectation if merely the distribution of chromosomes during fertilization is considered, is presumably due to the occurrence of random mutations in the germ cells, which prevent a complete homogeneity between the various individuals of an inbred strain. However, these mutations are able merely to delay but not entirely to prevent an approach to a completely homozygous condition, although this desired endstage, on account of such mutations, may never be fully reached by close inbreeding. There was, however, a difference in the rapidity with which progress in the direction towards this endstage was accomplished; it was more rapid in inbred guinea pigs than in inbred rats. In rats, a selection was made of the strongest members of each litter for the purpose of propagation. Such a selection was not made in the case of the guinea pigs. But, it is not probable that this factor would have been so influential as to cause the difference between the results of inbreeding in these two species. Such a difference may possibly be due to differences in the rate of mutations in guinea pig and rat. There is no indication that the sensitiveness of the host for and his reactivity against homoiogenous individuality differentials is greater in the rat than in the guinea pig.

Conclusion

In the course of more than one hundred consecutive brother-sister matings in family A, and of somewhat less than one hundred brother-sister matings in family B of King's inbred albino rats, a gradual increase in the homozygous condition of the inbred families has taken place, as indicated by the lessened severity of the reactions of the hosts against the individuality differentials of various members of these families. However, the advance was slight in the course of the first 67 generations of brother-sister matings; it was greater between the 67th and the 102nd generations; but even at the latter point a completely homozygous condition in the inbred family A had not yet been attained. These results were obtained in testing the interaction of the individuality differentials in litter mates, in members of different litters, as well as in hybrids of these inbred strains.

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FACTORS INFLUENCING GROWTH AND METAMORPHOSIS OF THE SALIVARY GLAND IN DROSOPHILA

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INTRODUCTION

The present investigations are concerned with the development of the salivary glands in Drosophila. The larval salivary glands are strictly larval organs and grow by increase in cell size. They are completely histolysed during the early part of pupal life. The salivary glands of the adult fly, on the other hand, are imaginal organs which develop from imaginal discs situated at the extreme proximal end of the larval salivary gland, and grow by cell multiplication. Thus both larval and imaginal salivary glands undergo a period of growth during larval life, while metamorphosis leads to the differentiation of the imaginal and the destruction of the larval salivary glands. From the work of Hadorn (1937) we know that the principle causing pupation in Drosophila is a hormone released by the ring gland, a small glandular organ situated dorsally between the two hemispheres of the larval brain. The role of the ring gland in the further differentiation of the various organs involved in the process of metamorphosis is, however, still obscure. Furthermore, virtually nothing is known about the causal factors concerned in the growth of organs during larval life. The present study attempts to analyze some of the causes which underlie the visible expression of growth and differentiation in the development of the salivary glands.

MATERIAL AND METHODS

Drosophila virilis was used for this investigation. Experimental animals were kept at a constant temperature of 25° ± 0.5° C. Instead of the usual method of transplanting larval tissues into larvae, the body of the adult fly was used as a carrier of the larval transplant. The larval tissues transplanted into the body cavity of the adult fly live in their new environment for a long time, perhaps indefinitely. They do not lose their developmental potencies; they grow and differentiate normally when provided with the appropriate stimulus. The adult hosts withstand the operation, which is simple in method, very well. The mortality rate is negligible even when the same host is used for continued transplantations. Indeed this new method approximates tissue culture more closely than any other so far known for insects. Its great advantage lies in the fact that one is able to study the developmental behavior of larval tissues outside their own larval environment. All histological observations on the salivary gland are based on orcein-stained material which was examined either in total mounts or in smear preparations. For certain developmental characteristics the glands were also examined in the living condition.

* Fellow of the John Simon Guggenheim Memorial Foundation.

It gives me great pleasure to extend my sincerest thanks to Drs. L. C. Dunn, Th. Dobzhansky, F. Schrader and S. Hughes-Schrader for many stimulating discussions concerning this work and for their continued interest in the progress of these investigations. I also wish to express my appreciation of the assistance given by my wife.

Normal Development of the Salivary Gland

In the normal development of the salivary gland one is confronted with a sequence of developmental steps, each of which is characteristic for a definite stage of development. Since the normal development of the salivary gland is necessary for the understanding of the experimental part of this paper, it will be discussed briefly. However, only such features of development will be set forth as are of importance for our special problem.

The salivary glands are paired organs. They develop as invaginations on either side of the anterior ectoderm. As development proceeds these invaginations elongate, grow inward, and unite medially into a common duct leading to the pharyngeal cavity. (Sonnenblick, 1940.) When the embryo hatches, the number of cells in each gland is, according to Makino (1938), about 115 and it does not change during larval life. The growth of the larval gland is, as already mentioned, the result of increase in cell size rather than cell multiplication. The development of the salivary gland has been staged from the time the larva leaves the egg to the time the gland is histolysed. Eleven successive stages of development are shown in Plate I, which represent photomicrographs of salivary gland total mounts, stained with orcein. In Plate II the distal portion of the same glands is shown at a higher magnification. Camera lucida drawings at a magnification of 85× were made for each gland of this normal series, as well as for the glands used in the experiments, and their circumference measured with a planimeter. The size values thus obtained together with special histological landmarks characteristic for the different developmental stages, assured great accuracy in the determination of the various stages. In the following the measurements of the circumferences are given for the normal series of development.

stage of development	1	2	1st molt	3	4	2nd molt	5	6	7	8	9	10–11
circumference (× 85) in cms.	2.5	4.0	6.0	9.0	11.0	12.5	14.0	17.0	23.0	28.0	41.0	meta.

The histological changes that occur during the development of the salivary gland are now set forth. The observations are based on orcein-stained total mounts.

Stage 1 (Plate I, Figure 1 and Plate II, Figure 1′).

The cell size is uniform throughout the gland. The cells are very small and the cell borders not clear. No details are visible in the nucleus.

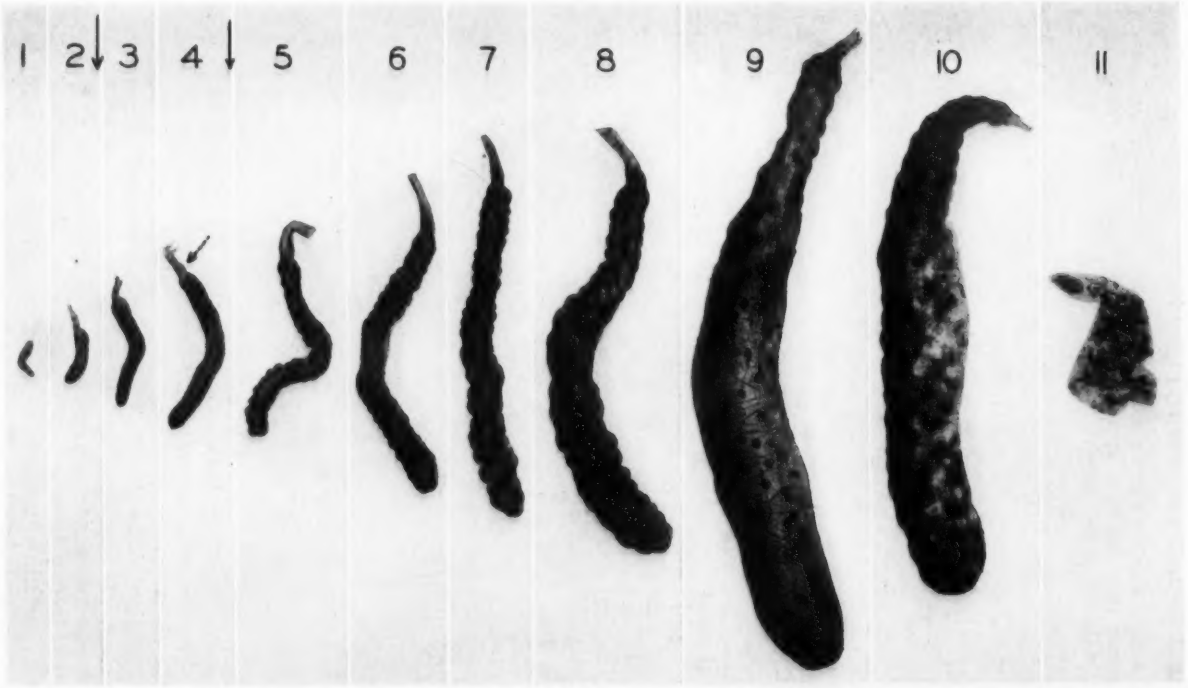

PLATE I. Normal developmental series of the larval salivary gland of Drosophila virilis. Figures 1 to 11: Photomicrographs of 11 successive stages of development. (Orcein stained total mounts.) Full arrows indicate time of molting. Broken arrow shows position of imaginal salivary gland anlage. (For explanation see text.)

Stage 2 (Plate I, Figure 2 and Plate II, Figure 2′).

The cell size is uniform throughout the gland. The chromatin forms a fine net-work around the edge of a clear area in the nucleus. The chromocenter is visible in all cells and stains deeply.

First molt. The cells are larger than in stage 2; otherwise there is no change.

Stage 3 (Plate I, Figure 3 and Plate II, Figure 3′).

The cells are larger; otherwise unchanged.

Stage 4 (Plate I, Figure 4 and Plate II, Figure 4′).

The cells are larger; they are still uniform in size throughout the gland. The chromatin forms a net-work consisting of fine strands around the edge of a clear area in the nucleus. The chromocenter is visible in all cells and stains deeply. At the proximal tip of the gland, where it borders the duct, a small number of tiny cells becomes visible; these represent the imaginal anlage of the adult salivary gland. (See Plate I, Figure 4, arrow.)

Second molt. The cells in the distal portion of the gland have become larger in size than those of the proximal portion. The chromosome strands, especially those in the nuclei of the distal gland portion, have become wider; alternating deeply stained areas and lightly stained areas are clearly distinguishable within the individual strands. The deeply staining chromocenter is still visible in all cells. The imaginal anlage of the adult gland has become very clear and its cells form a ring-like structure around the salivary duct.

Stage 5 (Plate I, Figure 5 and Plate II, Figure 5′).

The cells in the proximal gland portion are much smaller than in the distal; this difference is very pronounced. The chromosome strands have become wider and the chromatin bands more distinct. The chromosome strands on the periphery of the nucleus embrace the clear area in the center of the nucleus like octopus arms. The chromocenter is still visible in all cells. The imaginal ring cells have increased in number.

Stage 6 (Plate I, Figure 6).

The cells are larger; otherwise unchanged.

Stage 7 (Plate I, Figure 7 and Plate II, Figure 7′).

The difference in cell size between the proximal and distal gland portions is very clear. The chromosome strands are broad and the chromatin bands very distinct. The chromocenter has become invisible in the cells of about one-half of the distal portion of the gland. To avoid misunderstanding this point has to be made clear. The staining capacity of the chromocenter at this stage has by no means changed considerably, as smear preparations show. It has become invisible in total mounts because the chromosome strands in the distal cells have grown so much in width as to eliminate the former discrepancy in width between chromocenter and strands. Therefore if we speak in the following of the disappearance of the chromosome center, we mean just this fact. The disappearance

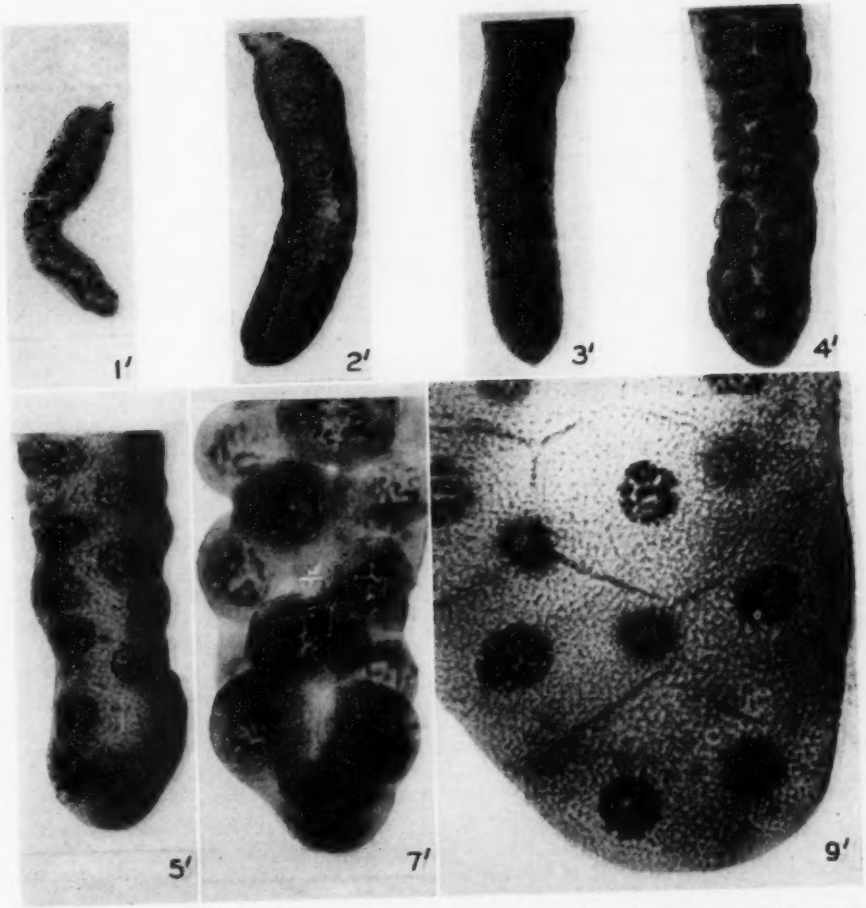

PLATE II. Figures 1′ to 9′: the distal portion of the same glands as shown in Plate I, Figure, 1 at a higher magnification. (For explanation see text.)

of the chromocenter is hence a good indication of the nuclear size. The imaginal ring cells have increased in number.

Stage 8 (Plate I, Figure 8).

The difference between the cell size in the proximal and distal part of the gland is very pronounced. The proximal cells have reached a size which corresponds approximately to the cell size in the distal part of the preceding stage. At no time, however, do we find any sharp separation into a proximal and distal portion of the gland as far as cell size is concerned. On the contrary, the cells increase gradually in size in a proximal-distal direction. Because of this differential growth, the salivary gland acquires its characteristic shape. The form and size of the salivary gland seem thus to be determined by two different growth rates in two different directions; one correlated with age affects the gland as a whole, and the other constitutes a proximal-distal gradient which determines the size of the cells throughout the length of the gland. While up to this time cell growth took place by an increase in size of the nucleus and cytoplasm simultaneously, at this stage a remarkable change occurs in the cells of the distal half of the gland. The nuclei in these large distal cells cease to grow, or grow only very little indeed, yet the cytoplasm increases immensely. A comparison of (Plate II) Figure 7′ with 9′ illustrates this point quite clearly. The chromocenter has become invisible in the distal two-thirds of the gland and the chromatin bands in the chromosome strands especially of the distal cells stain very distinctly. The imaginal ring has increased in size.

Stage 9 (Plate I, Figure 9 and Plate II, Figure 9′).

The larvae are full grown, they have left the food and are ready to pupate. In the living condition the cytoplasm of the glands has become slightly opaque instead of being transparent as in previous stages; this condition is the first indication of metamorphosis. The opaqueness is usually somewhat more pronounced distally. The proximal cells are still smaller than the distal ones. The cells in the distal two-thirds of the gland are extremely large, due to an increase in their cytoplasm, while their nuclei are of about the same size as in stage 8. Although the nuclei are unaltered in size, their condition has changed. The clear area in the center of the nucleus has disappeared. The chromosome strands have become more compact, winding their way through the whole nucleus. The individual strands show their characteristic banding most perfectly. The cytoplasm stains less heavily than in previous stages, while the nuclei take the stain exceedingly well. One of the characteristic features of this stage is that in total mounts the nuclei stand out clearly against a lightly stained background (Figures 9 and 9′). The chromocenters are visible only in the proximal one-fifth portion of the gland. The imaginal ring has become larger; the chromosome strands stretch very well in smear preparations, in contrast to earlier or later stages. This stage is hence best suited for studies on the arrangement of bands in the chromosome strands.

Stage 10 (Plate I, Figure 10).

Histolysis of the glands begins about 10 hours after puparium formation. In various regions, usually first in the distal part of the gland, the cells begin to

vacuolate and the cell walls rupture, while the nuclei are still intact. The cytoplasm in these regions of degeneration stain poorly. The chromosome strands on the other hand stain very deeply. They are clumped in the center of the nucleus and are surrounded by a clear spherical area (Figure 10). In living conditions the glands appear much more opaque, while milky-white zones indicate the regions of advanced histolysis. The chromocenter is by now visible only in a few cells at the extreme proximal end of the gland. The imaginal ring has increased in size.

Stage 10–11 (Plate I, Figure 11).

The next stages of histolysis proceed very rapidly. The degenerating regions within the gland which stain poorly, and which in life appear milky, extend and become more frequent. The nuclei become picnotic. The basement membrane which surrounds the gland breaks down. Finally, about 25 hours after puparium formation, with the probable help of phagocytosis, the larval gland is dissolved. The proximal part is the last to disappear. The differentiation of the imaginal ring begins and is completed during the rest of the pupal period, leading to the formation of the adult salivary gland.

Experiments

The induction of premature metamorphosis.

The description of normal development has revealed that the metamorphosis of the salivary gland consists of the histolysis of the larval and the differentiation of the imaginal salivary gland. The question now arises whether the characteristic developmental behavior of the gland during metamorphosis is dependent on or independent of the conditions prevailing in the animal during metamorphosis. This can be tested by transplanting young salivary glands into older hosts, thus exposing the gland prematurely to the metamorphosis factors.

Salivary glands of stage 5 were transplanted into the abdomen of older larvae shortly before pupation. The transplanted glands were dissected and their condition studied after they had remained in the hosts for various lengths of time. The results of the experiments are summarized in Table I A. The first animals in this series were dissected two days after the operation, when the hosts were about one-day old pupae. The transplanted glands had reached stage 10, i.e. they showed clear signs of histolysis. Their state of development corresponded closely to that of the normal host gland. Yet if the transplanted glands had been left in their own environment, they would have developed certainly not further than stage 8 by this time. Thus, under the influence of the metamorphosis factors of the host, the grafted glands have metamorphosed prematurely. The transplanted glands reached an advanced stage of histolysis when they were left three days in the host, and they disappeared completely when left in the host for six days or longer.

In these transplantations the anlage of the imaginal ring was included in the larval graft. Now it was found (Table I A), that the imaginal gland, like the larval gland, is able to metamorphose precociously when exposed to the metamorphosis factors prematurely. This becomes evident from the observation that the transplanted imaginal anlage in the newly emerged host, i.e. six to seven

days after the operation, is already completely differentiated into the imaginal salivary gland (Plate III, Figure 4), while donor controls by this time would have been two days old pupae with quite undifferentiated imaginal glands.

In summarizing the experiments at this point, we find that the metamorphosis of purely larval structures, as well as imaginal structures, is not autonomous,

TABLE I A

Transplantation of young salivary glands into larvae shortly before pupation.

A. Transplantations of larval glands of stage 5.

Circumference of transplanted gland	Days transplant remains in host	Host stage dissected:	Stage of transplanted larval gland	Stage of transplanted adult gland
14.0	2	pupa	stage 10+	larval
15.0	2	pupa	stage 10−	larval
15.0	2	pupa	stage 10+	larval
15.5	2	pupa	stage 10−	larval
15.5	2	pupa	stage 10−	larval
13.5	3	pupa	stage 11−	larval
13.5	3	pupa	stage 11−	larval
15.5	3	pupa	stage 11+	larval
15.5	3	pupa	stage 11−	larval
15.5	3	pupa	stage 11−	larval
13.5	6	adult	completely histolysed	adult gland formed
13.5	7	adult	completely histolysed	adult gland formed
14.0	7	adult	completely histolysed	adult gland formed
13.5	8	adult	completely histolysed	adult gland formed
13.5	10	adult	completely histolysed	adult gland formed
15.5	13	adult	completely histolysed	adult gland formed

TABLE I B

B. Transplantations of larval glands of stage 3.

7.5	2	pupa	stage 6	larval
7.5	3	pupa	stage 10−	larval
7.5	3	pupa	stage 10−	larval
9.0	3	pupa	stage 11	larval
7.5	6	adult	stage 11−	larval
7.5	6	adult	stage 11−	larval
7.5	6	adult	stage 11	larval
7.5	7	adult	stage 11−	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
7.5	10	adult	stage 11	larval
7.5	15	adult	stage 11	larval

but is caused by some factors in the organic environment of the host during the period of metamorphosis.

The next question to be answered is whether salivary glands younger than stage 5 are competent to respond to the metamorphosis factors. For this,

salivary glands of stage 3 were transplanted into older larvae shortly before pupation. The region of the imaginal discs was again included in the graft, but in contrast to the previous series of experiments the imaginal ring as such was not yet morphologically visible. The results of these experiments summarized in Table I B show that histolysis of the young transplanted glands begins about two days after the operation, but is never completed, although the glands may remain as long as 15 days in the host. Young glands which have remained for three days in the host only begin their histolysis, while older glands are at this time quite extensively histolysed (Cff. Table I A with I B). Thus the young glands are presumably unable to respond at a time when the metamorphosis factors are most effective. We must assume that the metamorphosis factors have become less efficient when the glands have finally reached their responsive stage, and are hence unable to induce complete metamorphosis, because it is difficult to understand why the glands should not be histolysed completely if the metamorphosis factors were still active in the late pupal or adult stage.

The inability of the young organ to respond to the metamorphosis factor is demonstrated in the behavior of the adult gland (Table I B). In spite of the fact that the young imaginal gland discs have remained in some cases for a considerable length of time in the host, they show no signs of metamorphosis (Plate III, Figure 3). Although the anlagen have developed well beyond their stage of transplantation and have acquired their typical ring-shaped form, they remain larval and never surpass this stage. Their state of development corresponds to stages 10 and 11 of the normal developmental series, yet this stage, as previous experiments have shown, by far exceeds the stage at which the adult gland anlage is able to react. These observations lead necessarily to the conclusion that the larval development of the disc is not interrupted in its new environment, but that by the time the disc has reached its reactive stage the factors necessary for promoting metamorphosis are absent or not effective enough. The specific results of this experimental series may then be briefly summarized as follows: the larval glands, as well as the analgen of the adult glands, must reach a certain developmental stage before they are able to respond to the metamorphosis factors. If they reach this stage after the active period of metamorphosis they are unable to metamorphose and persist as larval structures in an imaginal environment (Plate III, Figure 3).

In examining prematurely-metamorphosed young salivary glands, one has the impression that the nuclei in these glands are histolysed before they have attained their fully normal size. The same observation was made in prematurely-metamorphosed young salivary glands which were grown in the body of adult hosts. It is difficult to decide whether this impression is real, since the reduction of the nuclear size is only slight and probably within the limit of normal variation. If true, however, it would mean that premature metamorphosis causes an early cessation of nuclear growth, which is not at all unlikely. In the light of these considerations, it seemed interesting to investigate whether older glands transplanted into younger hosts would grow larger than normally, because under these experimental conditions they would be exposed to the metamorphosis factor later than normally. Salivary glands of stage 7 were therefore transplanted into younger host larvae, the salivary glands of which were at stage 4 at the time of the operation. In other words, salivary glands of the last larval instar were

transplanted into hosts of the second larval instar. This experimental series consisted of six hosts which were dissected two days after the operation when the transplanted and host salivary glands were compared. It was found that the host salivary glands had developed to stage 8−. They were still transparent, and showed no signs of metamorphosis. The transplanted glands on the other hand were found to be at stage 9+ and had begun to metamorphose, as indicated by their intensely opaque appearance. Moreover, the chromosome strands of the transplanted gland were distinctly wider than those of the host glands. Yet the strands were definitely not larger than normally, i.e. their size was characteristic for a normal salivary gland of stage 9+. Thus the original question whether it is possible to obtain salivary glands larger than normal has been answered by this experiment in a negative way. Some further conclusions which might be drawn here will be discussed on page 30 in a somewhat different connection.

The effect of the ring gland on the development of the salivary gland.

Until now we have dealt almost entirely with specific reactions of the salivary gland. So far nothing has been said about the factors which govern the processes of growth of the gland during larval life and those of metamorphosis during pupation. In the light of our knowledge of hormone-controlled insect metamorphosis, we expect these factors to be hormonal in nature. The search for the activating principle resolves itself into the problem of locating an organ or organs of internal secretion and demonstrating their action on the developing organ system. There are obviously two alternatives in attacking this problem experimentally. One may attempt to remove systematically various organs of supposedly endocrine nature from the larvae and thus hope to locate the responsible organ by testing the developmental behavior of the operated animal. The difficulty involved in a study of this kind is mainly a technical one, for it is very difficult indeed and at the present time seemingly impossible to perform such an operation in the larvae of Drosophila. The other possible approach open to the investigator is to dissect various organs from a donor larva which can be sacrificed and transplant them to a second larva and then test their effect on the development of the host. Although technically quite feasible, this procedure has one great disadvantage. The effect of the transplanted gland on the host may be counteracted or blurred to a great extent by the hormone supply of the host itself, and thus not detectable. The introduced hormone, moreover, may upset the host system to such an extent that the situation, instead of being clarified, is rather obscured. Apart from these difficulties there is the additional one that both approaches are rather indirect, since the experiments are designed

PLATE III, FIGURE 3. Adult salivary gland, developed from the imaginal salivary gland anlage which was transplanted at stage 6 into a host larva shortly before pupation. Transplant dissected from the emerged host six days after the operation.

FIGURE 4. Larval salivary gland persisting as larval structure in the adult host; obtained by transplanting salivary glands at stage 3 into the abdomen of host larvae shortly before pupation. Note: undifferentiated imaginal ring cells (arrows).

FIGURE 5. Paired salivary gland transplantation. Glands transplanted at stage 6 into adult hosts and dissected two days after the operation. *a*. Partner transplanted alone: note bloated appearance of gland and the small nuclear size. *b*. Other partner transplanted together with two ring glands. Note the large, very well stained nuclei with chromosome strands.

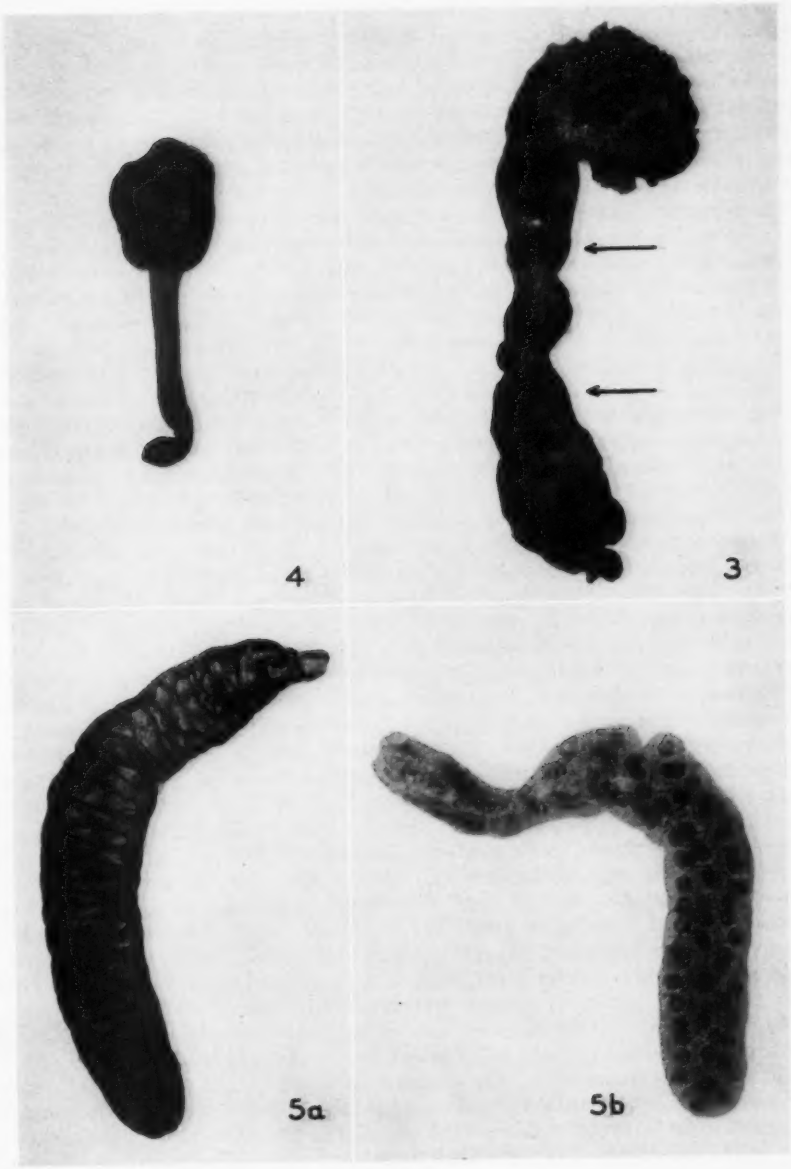

PLATE III

for testing the hormone action on the whole organism rather than on the special organ. We have hence to seek a method by which the hormone action may be more directly studied.

An effort in this direction was made by using the abdominal cavity of the adult male fly as a place for culturing larval tissue in vitro. This environment is supposedly neutral as far as the progress of development is concerned, for the larval salivary glands cease to grow after the transplantation, but continue to live indefinitely. The transplanted tissues do not suffer from a lack of nutrition nor are they unable to utilize the nutritive components of the new environment, as is shown by the following experiment. When young Drosophila larvae are starved, they use the fat stored in their fat bodies and, if starvation is continued, they exhaust their food reserves in the fat bodies completely. Such exhausted fat bodies are strikingly different from the fat bodies of feeding larvae. Now it was found that starved exhausted larval fat bodies restore their food reserves again and become indistinguishable from fat bodies of normally fed larvae when transplanted into the body cavity of adult flies. For these experiments a small strip of fat body closely attached to the salivary gland was transplanted together with the gland. The gland, important in this instance only as a marker, enables one to find and distinguish the transplanted fat body from the fat bodies of the host. The inability of the larval organs to develop in the adult fly is hence not caused by a lack of nutrition but obviously by the lack of some other factor.

Before considering the nature of this developmental factor, one further point has to be cleared up. It has to be shown whether the inability of the gland to develop is typical for glands of all ages or only characteristic for glands of a particular stage of development. In order to elucidate this point, salivary glands of stages 2 to 6 were transplanted into the abdomen of adult male flies. The grafted organs were allowed to remain for various lengths of time in the host before they were dissected out and their developmental condition examined. The results of these experiments summarized in Table II show clearly the inability of the gland to develop in the body of the adult fly regardless of their age at the time of the transplantation and of the length of time they remained in the host. The only perceptible way in which the transplanted glands seemed to be changed while in the adult host was that they became greatly inflated, this being caused by the accumulation of a clear watery fluid in the lumen of the gland. The longer the glands remained in the host, the more fluid accumulated and the more bloated the glands became. The accumulated fluid presumably represents saliva, which is secreted by the gland cells into the lumen of the gland and is unable to escape, since the outlet is closed off in dissecting the gland from the donor larvae. Incidentally, should this fluid really prove to be saliva, we have here a method of accumulating it in order to study its chemical properties.

The foregoing experiments have shown that the larval salivary glands depend for their development upon some factor missing in the body cavity of the adult fly. This factor was found to be the larval ring gland, which when transplanted simultaneously with the salivary glands into the abdominal cavity of the adult fly, caused the latter to continue their development leading to histolysis. The progress of development of the salivary glands was indicated by changes in the nuclei, cell growth and certain characteristic staining reactions. All the typical developmental stages found and described in the normal developmental series

may be observed, but they proceed at a much lower rate. The salivary glands are also able to metamorphose, i.e. they become gradually histolysed if left long enough in the adult host. The ring glands used for these experiments came from donor larvae which were ready to pupate. The number of ring glands implanted into one host varied from one to four. Salivary glands of stage 2 to stage 6 were tested for their reaction to the ring gland factor. The transplanted salivary glands remained in the host for various lengths of time. These experiments, summarized in Table II, led to the discovery of a number of essential facts concerning the development of the salivary glands. Glands of all ages tested respond to the ring gland factor. The older the gland is at the time of transplantation, the earlier metamorphosis occurs. This implies that at least younger glands do not metamorphose right away, but grow to a certain point before metamorphosis begins. In the study of normal development we have seen that the first signs of metamorphosis are noticeable in glands of stage 9, as indicated by a slight but distinct opaqueness of the gland in living condition. At this time the chromocenter has become invisible in the distal portion of the gland. We find in glands which develop in adult hosts some variability in the relationship between the onset of opaqueness, i.e. metamorphosis, and the disappearance of the chromocenter. The degree of opaqueness observed in the transplanted glands is indicated in Table II by the Roman numerals I to III placed next to the number which designates the stage of development. The number I represents an opaque condition of stage 9 and the number III of stage 11− in normal development. When no Roman numerals are given, the relation between metamorphosis and developmental stage is normal. The transplanted glands become opaque at an earlier developmental stage. This is especially true in the transplantation of younger glands. For instance, glands which have developed in the adult host to stage 7− show an opaque condition which corresponds to stage 9 or 10 in normal development. Since we know from normal development that the chromocenter becomes invisible because of the thickening of the chromatin threads, it follows that the glands begin their metamorphosis before the strands have developed to their normal size. It was also noticed in many cases that the nuclear size in advanced prematurely metamorphosed glands seemed smaller than normal. This would indicate that they degenerated before they had reached their full size. These facts are in agreement with the experiments described on page 19 where young glands in old larval hosts were made to metamorphose precociously. The number of ring glands transplanted seems to be of no great importance for the development of the salivary gland, since one or three ring glands produce about the same effect.

In all these experiments where adult flies were used as hosts the imaginal salivary anlage usually transplanted together with the larval gland remained larval. Its cell number, however, increased considerably when transplanted together with ring glands. The amount of increase depended upon the time the anlage remained in the host. However, no sign of differentiation could be noticed, in spite of the fact that in some cases more than four ring glands were present and the disc remained for a considerable length of time in the host. In the effort to obtain a more precise comparison between the developmental behavior of larval salivary glands with and without ring glands, the two glands of one donor were compared with each other. For this experiment the paired

gland of a single donor was dissected, the two glands separated and one partner transplanted into one adult host without ring glands and the other partner transplanted into a second adult host together with two to four ring glands.

TABLE II

Transplantation of salivary glands of various ages; alone and together with different numbers of ring glands into the abdomen of adult male flies. Roman numerals indicate state of opaqueness of the gland. (For explanation see text.)

Stage of transplanted salivary gland	Days transplant remains in host	Number of ring glands transplanted									
		None		One		Two		Three		Four	
		Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development
	2	1	3−	1	I 9						
2	3			2	5						
↓	4	2	3	2	I 7−			1	5		
2	5									1	4+
	7	2	3−					2	6	1	I 7−
	14	1	3−					1	II 9+		
	6	1	3			1	I 7+				
3	9	2	3			2	II 8				
	13	1	3			1	III 10				
	1	1	4					1	5+		
4	4	4	3+	1	I 7			1	I 8−		
↓	6	4	4−	2	I 7					3	10
4	7	4	4−					2	II 8+		
	8	3	4+								
	9	2	4+							1	II 8
	2	1	5−					3	6		
	2	1	5					2	II 9		
	5	1	5							1	I 7
5	6	1	5			1	8				
	7							1	II 9−		
	9	2	5			2	10+				
	10	1	5					1	10		
	13	1	5−			1	11−	1	11−		
	2	5	6			5	9+				
6	4	1	6			1	10−	1	III 9+		
	7	1	6			1	10+	2	10		
	14							1	11−		
Number of cases		45		8		15		20		7	

The gland in the first host thus served as a control for the partner gland in the second host. At the desired time, both hosts were killed simultaneously, the two glands dissected and compared. Twenty-three such pairs are available;

they are recorded in Table III. As in the previous experiments we find the salivary glands unable to develop when transplanted alone, while the partner glands with added ring gland implants continue development. Figure 5 of plate III illustrates such a paired transplantation. This pair was transplanted at stage 6 and left for two days in the adult host. The partner (*a*) transplanted alone, has become greatly inflated, yet has not developed beyond the stage of transplantation. The other partner (*b*) which was transplanted together with

TABLE III

Paired transplantation of salivary glands of different age into the abdomen of adult male flies. Note: one host carries salivary gland alone, while second host carries partner salivary gland and ring gland grafts.

Stage of transplanted salivary gland	Number of ring glands transplanted	Days graft remains in host	Condition of transplanted salivary gland	
			Partner without ring gland stage:	Partner with ring gland stage:
2	4	5	2	4+
3	2	6	3	I 7+
3	2	9	3	II 8
3	2	9	3	II 9
3	2	13	3	10
4	3	1	4	5+
4	3	4	4	I 8−
4	3	7	4+	II 8+
4	3	7	4	III 9+
5	2	6	5	II 9+
5	2	9	5	10−
5	2	9	5+	10+
5	2	13	5	11−
6	2	2	6	9+
6	2	2	6	9+
6	2	2	6+	9+
6	2	2	6	9+
6	2	2	6	9+
6	2	2	6	9+
6	2	3	6	9+
6	2	4	6	10−
6	2	6	6	10+
6	2	7	6	10+

two ring glands has continued its development and has reached stage 9+. Plate III, Figure 5b illustrates clearly the characteristic large nuclei at this stage which stand out against a lightly stained background (Cf. Plate III, Figure 5b with stage 9 of the normal developmental series). The chromocenter has become invisible in at least two-thirds of the gland. The chromosome strands are broad and the chromatin bands within them very distinct. The single cells are extremely large, due to an increase in the volume of the cytoplasm. In the living condition the cytoplasm is opaque in contrast to the transparent

appearance of the partner gland. Moreover, the salivary gland which has undergone development is not swollen as is the case with the partner gland. This fact can be seen in all salivary glands which are transplanted together with ring glands. The ring gland must hence interfere with the secretory function of the salivary gland. On turning again to Table III we find that the first response to the ring gland is obviously a growth response. This becomes evident if one compares glands of the same age which were left for increasingly longer periods in the host. (See Tables II and III.) For example, glands transplanted at stage 4 grow in one day to stage 5+, in four days to stage 8− and reach stage 9+ when left for seven days in the host. After growth has continued for a certain length of time, the physiological condition of the glands must have changed in some way, since they now respond with metamorphosis to the ring gland factor. The younger the glands are at the time of transplantation, the later they respond with metamorphosis, for glands transplanted at stage 2 develop in five days only to stage 4+, while glands transplanted at stage 4 show indications of metamorphosis after four days. Metamorphosis is far advanced in four days when glands of stage 6 are used for the transplantation. The transplanted glands, especially the younger ones, metamorphose precociously, for the opaque metamorphosis condition is noticeable in transplanted glands as early as stage 7+, thus at a considerable earlier stage than in normal development. (See also Table II.) This abnormal relationship between the stage of development and the onset of metamorphosis becomes less pronounced as increasingly older glands are used.

The different responsiveness of younger and older salivary glands has been demonstrated very clearly in a further experiment especially designed for testing this point. Two salivary glands, one of stage 3 and the other of stage 5, were transplanted together with two ring glands into the abdomen of a single adult host. This series consisted of four hosts, each of which contained a young and an old salivary gland, as well as two ring glands. Six days after the operation, the first host was examined. The young salivary gland had developed to stage 7+ and was slightly opaque (I), while the older gland was at stage 9+ and distinctly more opaque. In two hosts examined nine days after the operation, the younger glands were found to be at stage 8 to 9 and the older glands at stage 10+. While the younger glands appeared intensely opaque, the older glands were obviously in an advanced state of metamorphosis, as indicated by large milky regions of degeneration. The last host examined 13 days after the operation revealed the young gland at stage 10 and the older one at stage 10+. Again histolysis had progressed further in the older gland.

In a second series of this kind, consisting of six animals, salivary glands of stages 2 and 3 were transplanted together with three ring glands into a single host. The results of these experiments are essentially the same as in the foregoing series; they show again that older glands are always further metamorphosed than young ones, although, as pointed out before, the onset of metamorphosis in the young glands is premature, relative to their state of development.

It has been seen that the development of the salivary gland in the adult host with the support of the ring gland is considerably slower than in normal development. However, the question as to how much development is slowed down has not as yet been examined. In order to elucidate this point, one partner

of a pair of glands of stage 5 was transplanted into the abdomen of an adult host together with 3 ring glands, while the other partner was transplanted into the abdomen of an old larva shortly before pupation. In this way it was possible to make a direct comparison of the same gland in an adult and larval environment. From six such experimental pairs, five were examined two days after the operation. At this time the larval hosts pupated. In one pair the gland in the larval host was found to be at stage 10, showing clear regions of degeneration, while the partner gland in the adult host had only developed to stage 9 and appeared but slightly opaque. In two other pairs only the proximal part of the gland in the pupated larval host could be found, since the distal gland region was completely histolysed. The partner glands in the adult host in one of these pairs had developed to stage 9, being slightly opaque, while the gland in the other adult host had reached only stage 8 and was still transparent. The glands in the pupated larval hosts of the two remaining pairs could not be found, presumably because they were already completely histolysed. The partner glands in the adult hosts were found to be transparent and had reached stage 8. The last pair of this series was examined seven days after the operation. At this time the larval host had already emerged. Its graft could not be found and was apparently completely histolysed. The partner graft in the adult host was at stage 10−, showing advanced signs of histolysis.

The results of these experiments bring into focus one interesting point. We have seen from the previous experiments that growth and metamorphosis of the salivary gland is controlled by the ring gland. The salivary glands in the larval hosts are under the influence of only one ring gland, namely that of the host, while in the adult hosts the glands are exposed to the effects of two ring glands. In spite of this, we find the progress of development of the salivary glands much more rapid in the larval than in the adult host. This shows that the ring gland factor is more effective in a larval than in an adult environment.

Before closing the experimental part of this paper it should be mentioned that the ring gland factor is not species-specific, since ring glands of melanogaster cause the development of virilis salivary glands in both virilis and melanogaster hosts. Conversely, melanogaster salivary glands develop under the influence of virilis ring glands in virilis or melanogaster hosts.

Discussion

The present data prove without doubt the necessity of the ring gland for the metamorphosis of the salivary gland. The responsible ring gland factor is presumably hormonal in nature, since the transplanted ring glands have no contact with the test organs which are nevertheless compelled to metamorphose. The ring gland is thus the source of the metamorphosis hormone in Drosophila and is not responsible only for puparium formation as previously assumed. In fact, puparium formation has to be considered as the first step in metamorphosis. The observations of Hadorn and Neel (1938) that ring glands implanted into young larvae bring about premature puparium formation but have no effect on the metamorphosis of the larval organs can be explained by the incompetence of the young organs to respond to the metamorphosis hormones. This is supported by the observation that young salivary glands react more slowly to the ring gland hormone than older ones and that the salivary glands must reach a

certain developmental stage before they are able to respond to the metamorphosis hormone. The inability of organs to respond with metamorphosis before a certain stage of development is reached is not peculiar to salivary glands but has been observed for eye discs (Bodenstein, 1939a and b), and for ovaries (Vogt, 1941). Inasmuch as the time at which larval glands and imaginal gland anlagen reach their state of responsiveness is concerned, we find that both structures become responsive at about the same time. This, however, is true only of grafts into larval hosts; in adult hosts we find the larval glands able to metamorphose, but not the imaginal glands, in spite of the presence of several ring glands. This indicates clearly that the larval glands respond much more readily than the imaginal glands. In comparing the ring gland action in a larval and an adult environment, we find the hormone more active in the former. Whether one ring gland in the larval host is able to produce more hormone and thus assure a higher hormone concentration than four ring glands in an adult host is questionable, but would explain easily why in the adult host the imaginal anlage is unable and the larval gland is able to respond; and why in the larval host both glands are able to metamorphose synchronously. On such an hypothesis the low hormone level in the adult host is able to induce metamorphosis only in the readily responsive larval gland but not in the imaginal gland anlage, which needs a higher hormone concentration for the same task. In the larval environment on the other hand, where the hormone concentration is presumable higher, it is sufficient to induce metamorphosis in the imaginal gland also. In the light of these considerations it becomes evident that any difference in the competence of the reactive tissue might be detectable only when low hormone concentrations are used. The quantitative action of the metamorphosis hormone has also been demonstrated by Hadorn and Neel (1938), who found that pupation in Drosophila occurs sooner when three instead of one ring gland are implanted into the larval host. The question of hormone concentrations was not directly tested in our experiments because there seemed to be no difference in the effect of two or more ring glands. There is, however, some indication that one ring gland is not as effective as four; this can be seen if one compares (Table II) salivary glands which were transplanted at stage 4 together with one and with four ring glands and which were then left for six days in the adult host. The salivary glands (two cases) transplanted together with one ring gland have developed to stage 7 and the salivary glands transplanted together with four ring glands to stage 10 (three cases). Similar experiments on other organ discs not reported here gave the same results. Thus it seems quite certain that the hormone concentration is a decisive factor in the development of the salivary gland.

One of the most important facts revealed by the present investigations is that the first salivary gland response to the ring gland hormone is a growth response. It is only after the salivary glands have grown to a certain size that the hormone elicits the metamorphosis response in the salivary gland. The younger the salivary glands are at the time of transplantation the longer the growth period persists. In other words, younger glands begin their metamorphosis later than older glands when under the influence of the same number of ring glands, yet the onset of metamorphosis of young glands in adult hosts under ring gland influence is definitely premature when compared with normal develop-

ment. However young the salivary glands are when transplanted, the onset of their metamorphosis is never earlier than stage 7−, as compared with stage 9 in normal development. This shows that the young glands are able to respond with growth only they have reached stage 7−. This observation leads us directly to the question why in normal development the onset of metamorphosis takes place at stage 9 and not at stage 7−. The answer to this may be found in the following considerations. The experiments have shown that the growth of the salivary glands depends on the ring gland hormone. This must obviously also be the case in normal development. Now in normal development the salivary gland is under the influence of the ring gland during the entire larval period. However, the ring gland of younger larval stages is much smaller than the ring gland of mature larvae and thus probably produces much less hormone. The hormone concentration of younger larvae is consequently expected to be much lower than in older larvae. The hormone level in younger larvae, we might argue, is thus not high enough to assure metamorphosis, but is sufficient to promote growth. As development proceeds, the ring gland grows and thus produces more hormone; at the same time however, we find the competence of the salivary gland changing. At a given time, therefore, when hormone level and responsiveness of glands are in a definite relationship with each other, metamorphosis occurs. In normal development this time is reached when the salivary glands have developed to stage 9. We may now ask why, under experimental condition, we are able to metamorphose glands which have reached only stage 7−. The answer to this lies very probably in the fact that we have changed the hormone concentration. In the adult host the hormone level produced by several ring glands is presumably much higher than in younger larvae and is thus able to induce metamorphosis in glands as young as stage 7−. Since metamorphosis proceeds at a very slow rate, we must conclude that the experimentally established hormone level in adult animals, while higher than in younger larvae, is not as high as in full-grown larvae, where young glands metamorphose much more rapidly. Whether the ring glands produce less hormone in the adult fly, or whether some other factors are responsible for the fact that we are unable to raise the adult hormone level to the equivalent of the level existing in the old larvae is, however, still questionable.

There is one further point of considerable interest. We have seen on page 22 that old salivary glands transplanted into younger larvae metamorphose before the glands of the host larvae show any signs of metamorphosis. Since the cell size of the grafted glands was found to be not larger than normal, there seems to be a limit beyond which nuclear growth is impossible. The question now arises, what exactly do we mean by this limit? Judging from the results cited earlier, we have to assume that the salivary glands become more and more responsive to metamorphosis as they increase in age. The older gland in the young host is therefore at a much more advanced stage of responsiveness than the host glands, and is hence able to react with metamorphosis to a hormone level much lower than that needed for the same reaction by the younger gland. In assuming, as we have, that the hormone level increases gradually during the larval period, the growth limit is nothing more than the expression of a definite relationship between cell competence and hormone level. In the light of this it is theoretically possible to obtain cells larger than normal, when older glands

are affected by a hormone level too low for metamorphosis but high enough for growth; granted, however, that a hormone level low enough for a highly responsive gland could be found.

Logically this conception is based on the assumption that the ring gland produces its hormone during the entire larval life. Only with the demonstration of this does the present hypothesis become meaningful. Actually there is really good evidence available which shows that ring glands from larvae as young as the first instar are able to promote growth in certain organ discs. More precise information concerning this point will be given in a later communication.

Summary

1. The normal development of the salivary gland of *Drosophila virilis* is described. Eleven successive stages of development have been distinguished.

2. Larval salivary glands of different ages were transplanted into the abdomens of older larvae and thus exposed prematurely to the metamorphosis factor. It was found that the metamorphosis of the transplanted glands is not autonomous but depends upon some factor in the host.

3. Glands as young as stage 3 are unable to react to the metamorphosis factor and persist as larval tissue in the adult fly. However, glands transplanted at stage 5 metamorphose synchronously with the host and hence undergo a premature metamorphosis. In these cases the transplanted larval glands are completely histolysed, and the simultaneously transplanted anlagen of the imaginal salivary gland differentiate into adult salivary glands.

4. Salivary glands of older larval donors transplanted into younger hosts metamorphose before the host glands show any signs of metamorphosis.

5. Larval salivary glands of various ages were transplanted into the body cavity of adult male flies. The thus transplanted glands ceased to grow and remained unchanged even when left for a considerable length of time in their adult environment. If, however, ring glands of old larvae are transplanted together with salivary glands into the adult host, the growth of the salivary glands is restored, leading finally to metamorphosis. These facts have been demonstrated very clearly by using salivary glands of a single donor and transplanting one partner into one host without ring glands and the other partner into a second host together with ring glands.

6. The number of ring glands implanted is of no great importance for the development of the salivary glands, since two ring glands have about the same effect as four ring glands. However, one ring gland is presumably somewhat less effective than four ring glands.

7. The rate of metamorphosis of the salivary gland in adult hosts is decidedly slower than in normal development.

8. Younger salivary glands metamorphose later than older salivary glands under the influence of the same number of ring glands.

9. Although the young glands metamorphose later, their metamorphosis is premature as far as their state of development is concerned.

10. A comparison of the time of metamorphosis of salivary glands in adult and larval hosts shows that metamorphosis proceeds much more rapidly in larval hosts in spite of the fact that in the adult host the salivary glands may be under the influence of as many as four ring glands.

11. The ring gland factor is presumably hormonal in nature, and is not species-specific.

12. The role of hormone concentration and tissue competence in the determination of the various stages of growth and differentiation in the development of the salivary glands is discussed.

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HORMONES AND TISSUE COMPETENCE IN THE DEVELOPMENT OF DROSOPHILA

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The larvae of Drosophila molt twice, and change with the third molt into the pupal stage, during which the larval organism is gradually made over into the final adult insect. It was found (Bodenstein, 1936) that the initiation of pupation depends upon some factor in the anterior part of the larva which becomes active shortly before pupation and which is presumably hormonal in nature. Hadorn (1937) has located and analyzed this factor more precisely. He brought forward conclusive experimental evidence that a hormone causing pupation in Drosophila is produced by the ring gland, a small organ of internal secretion situated dorsally between the two brain hemispheres of the larvae. Although responsible for pupation, the ring gland was seemingly unable to initiate further pupal development, i.e. the differentiation of the larval organ anlagen to imaginal completion. For larval abdomens which, as a result of the removal of the anterior part, remain constantly larval could be caused to pupate when one or more ring glands were transplanted into them (Hadorn and Neel, 1938). Yet only puparium formation but no further development could be induced. Likewise, transplantation of several ring glands into younger larvae brought about only precocious puparium formation but again no subsequent development (Hadorn and Neel, 1938). In the light of these facts it appeared highly probable that some other hormone than that for puparium formation governed imaginal differentiation. The following observations seem to verify this assumption. The imaginal differentiation of pupal abdomens proceeds to imaginal completion when the anterior pupal part is cut off about 20 hours after pupation but the abdomen remains pupal when the anterior part is removed earlier (Bodenstein, 1938 and 1939a). The imaginal differentiation of organ anlagen, for example, eye discs, also depends upon this factor in the anterior part. On the basis of this rather indirect evidence a special differentiation hormone was postulated (Bodenstein, 1938). However, attempts to localize this factor in the anterior pupal part failed completely (Bodenstein, 1939a and c). Pupal abdomens, the anterior part of which was cut off before the imaginary hormone was released and which consequently were expected to remain pupal, continue their development to imaginal completion when placed in a pure oxygen atmosphere (Bodenstein, 1939c). This observation made the existence of a special hormone for differentiation very doubtful. Moreover it was shown (Bodenstein, 1939c), that the inability of the pupal abdomen to develop could be correlated with disturbances in the development of the tracheal system. These experiments, then, indicated that abnormalities in the functional development of the tracheal system rather than the lack of a special hormone was the cause of the inability of the abdomens

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to develop. While these considerations do not disprove the existence of a differentiation hormone completely, they make its assumption quite unnecessary. The main object of this paper is to bring forward more conclusive evidence that the ring gland is responsible not only for pupation but also for differentiation. Actually we have to consider pupation as the first step in the process of imaginal differentiation.

Material and Methods

The experiments reported here were performed on *Drosophila melanogaster* (Ore. R+) and *Drosophila virilis* (wild stock). Both of these species were used as donors and hosts. Various organ discs were transplanted into the body cavity of adult flies and the development of the transplant in its new environment was studied. This new method (see Bodenstein, 1943b, in press) of using the body cavity of adult flies as a culture medium for larval tissues proved to be very successful and was used throughout this investigation. The mortality rate in these experiments was negligible. All the experimental animals were kept at a constant temperature of 25° ± 0.5° C.

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Experiments

When larval eye or leg discs of Drosophila are transplanted into the body cavity of adult flies, the grafted organ is unable to develop. Although left for many days in the adult environment the graft remains unchanged as far as its morphological appearance is concerned. This observation is in agreement with earlier experiments of this kind (Bodenstein, 1938, p. 497). From this it was assumed that the adult environment is a medium unsuitable for the development of larval organs. It was therefore rather unexpected when it was found that larval eye discs which were transplanted simultaneously with two ring glands of mature larvae into adult flies had grown well beyond their original size. This experiment was repeated as follows: Eye discs of melanogaster donor larvae of equal age were transplanted either alone or together with ring glands into adult melanogaster hosts. Three days after the operation the grafted eye discs were dissected and compared. It was found that the eyes in hosts with ring glands were larger by far than those which were in hosts without ring glands. A great number of similar experiments was then performed, consisting of 243 cases where organ discs were transplanted together with ring glands into adult hosts and 156 control cases where the organ discs were transplanted alone. The bulk of this material comprises many different series; the series varied as to the time the organs were allowed to remain in the adult host, the number of ring glands transplanted into one host, the kind of organ disc used (eye and leg) and the kind of host used (melanogaster and virilis). In comparing the discs in hosts with and without ring glands it was invariably found that the organ discs in the hosts with ring glands had become much larger than the control discs in the hosts without ring glands. Further proof for the initiation of growth by the ring gland was obtained by experiments in which the two partners of a single

organ pair were compared one with the other. For this a pair of eye or leg discs was dissected from a single donor larva and one partner disc transplanted into one host together with two to four ring glands, and the other partner transplanted alone into a second host. The results of these experiments consisting of 34 individual pairs are summarized in Table I, where it can be seen

TABLE I

Paired transplantation of eye and leg discs into two adult hosts. One host receives disc alone, while other receives partner disc and two to four ring glands.

Transplant	Number of pairs	Days pairs remain in hosts	Number of pairs where the disc is larger in hosts with ring glands
mel. eye	3	2	3
mel. eye	1	3	1
mel. leg	4	3	4
mel. eye	3	4	3
viril. leg	2	4	2
mel. leg	2	5	2
viril. leg	8	5	8
mel. eye	1	6	1
mel. eye	1	8	1
mel. leg	1	8	1
viril. leg	3	9	3
viril. leg	1	12	1
viril. leg	1	14	1
viril. leg	3	16	3

that in each pair the disc which was transplanted together with ring glands had become larger than its partner. Figure 1 (*a*, *b*) illustrates very clearly the enormous size difference between two partner discs which were dissected and photographed five days after the operation. This particular pair is a melanogaster leg pair. One partner disc (*a*) was transplanted alone and the other

PLATE I

FIGURE 1. Melanogaster leg disc pair five days after the operation. One partner disc (*a*) was transplanted into an adult melanogaster host; the other partner disc (*b*) into an adult melanogaster host together with four ring glands. Note: the enormous difference in size between the two discs.

FIGURE 2. *b*: virilis leg disc four days after the operation, showing the first signs of metamorphosis, i.e. the beginning of evagination. The disc was transplanted together with four ring glands into an adult virilis male host. *a*: Virilis leg disc at the time of transplantation. *c*: a normal pre-pupal leg disc beginning to evaginate. Note: the similarity in the process of evagination between the transplanted (*b*) and the normal (*c*) leg.

FIGURE 3. A virilis leg disc transplanted together with three ring glands into an adult virilis female host, 13 days after the operation. The leg disc is completely differentiated. Note: the well-formed dark brown chitinous tarsus segments, with hairs and claws well developed.

FIGURE 4. Virilis leg disc pair transplanted into two adult virilis male hosts six days after the operation. Note: same size of both discs.

FIGURE 5. Virilis leg disc pair four days after the operation. One partner disc (*a*) was transplanted into an adult virilis male host and the other partner disc (*b*) into an adult virilis female host. Note: disc in female host (*b*) has become much larger than the partner disc (*a*) in the male host, which has not grown at all.

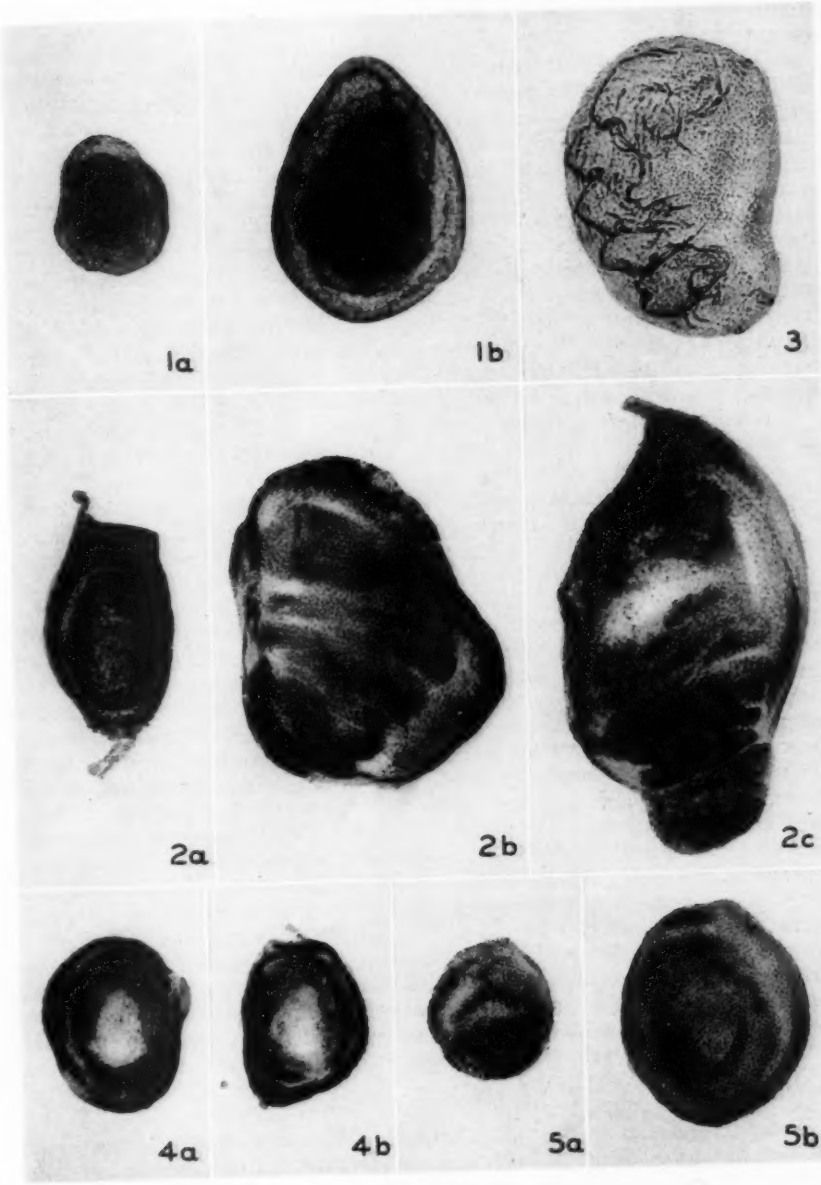

PLATE I

partner (*b*) together with four ring glands into the abdomen of adult melanogaster hosts.

In normal development we notice that a short time after puparium formation many organ discs undergo a characteristic change of form; they evaginate and obtain thus their typical pupal shape. This evagination process is one of the first visible signs of metamorphosis of the organ discs. Now we find that the transplanted leg discs in the body cavity of adult flies also evaginate under the influence of the ring gland after they have grown to a certain size. This induced evagination process is not quite complete, presumably because of mechanical difficulties, but is nevertheless very clear. This is illustrated in Figure 2 (*a*, *b*), Figure 2*a* shows a leg disc at the time of transplantation; Figure 2*b* a leg disc of a normal young pupa which has started to evaginate, and Figure 2*c* a disc which was transplanted into an adult host together with ring glands and which was dissected four days after the operation. In comparing Figure 2*b* with Figure 2*c* one may notice the similarity between normal and induced evagination. This observation clearly proves that the ring gland is able to induce the first stages of metamorphosis in the transplanted leg anlage. Moreover the ring gland is able to induce complete metamorphosis in the leg, if the leg is left in the adult host long enough. In these cases we find a completely differentiated imaginal leg with femur, tibia and tarsus segments as well as well-formed and dark brown chitinized hairs, bristles and claws in the abdominal cavity of the fly (Figure 3). From these experiments it becomes evident that the ring gland is not only responsible for an early initiation of growth, but also for the imaginal differentiation of the organ discs.

While it is true that organ discs transplanted together with ring glands were always larger than the control discs transplanted without ring glands, there was nevertheless a certain variability in the growth of the discs. In some cases where the discs were transplanted alone into adult hosts it was found that they had not grown at all, although they had remained for ten days or longer in these hosts. In other cases the discs had grown quite well and even showed signs of metamorphosis although no ring glands were present. Similarly, discs of equal age grown for the same length of time in the presence of the same number of ring glands could vary quite extensively in size. Now it has to be realized that in experiments of the kind described one deals with three different developmental systems which together determine the outcome of the experiment. These systems are: 1. The adult host environment; 2. The activating system, i.e. the ring glands; 3. The reacting system, i.e. the test organ discs. Thus in order to clarify the observed discrepancies in the experiments, a more thorough investigation of these three systems was undertaken. For this purpose experiments were designed in such a way that two of the systems were held constant and the third one varied. In doing this for each system in turn a clear understanding of the part played by each system was obtained.

I. The adult host environment

Under this general heading we will discuss a number of experiments in which the adult flies used as hosts were varied. As an indicator for possible differences between the various hosts we used only the early growth reaction of the test organs. This method is very sensitive, for even small differences reflect them-

selves very clearly in the growth of the test organ discs, especially when the two partners of a single organ pair are compared.

a. Growth variability test in male hosts without ring glands.

This series was designed to test whether there is any difference in the growth of test organs in different host individuals of the same sex. For this purpose, pairs of leg discs were dissected from virilis larvae and one partner disc transplanted into one and the other partner disc into a second adult virilis male host. The two hosts were then reared and dissected together. From eight such pairs two pairs were dissected two days, one pair three days and five pairs five days after the operation. The partner discs in all pairs were found to be the same size (Figures 4*a* and 4*b*). This proves that there is no detectable difference in the environment of the different individual male hosts as far as the test organs are concerned. Moreover, the leg discs remained unchanged in size, which indicates that no growth had occurred from the time of transplantation until they were dissected five days later. In order to obtain more information on this point, four of these disc pairs were again transplanted into adult male hosts. Seven days later they were dissected and found to be unchanged. Thus we must conclude that the virilis organ discs are unable to grow in an adult virilis male environment.

b. Growth tests in hosts of different age.

The question whether there is any difference in hosts of different age has been tested in the following way. The two partners of a pair of virilis leg discs were transplanted, one into a one-day old virilis male host and the other into a 29-day old virilis male host. From five such pairs one was dissected four days, and four pairs six days after the operation. In all cases it was found that the partner discs of the single pairs were of the same size. There was also no growth in either partner discs during the time they remained in the hosts.

In a second series consisting of six pairs, the discs were transplanted into female hosts instead of into male hosts. One female host was two days old and the other 30 days old. One pair, dissected three days and five pairs dissected five days after the operation revealed again that the two discs of one pair were of equal size. Yet in contrast to the previous series each of the discs had grown during the time it remained in the host.

These experiments prove that there is no difference between young and old hosts. They confirm the previous observation that the environment of each individual male host is the same, and extend the information in showing that this is also true for the environment of each individual female host. The observation that no growth takes place in discs transplanted into male hosts is also confirmed. However, when one compares female and male environment one finds the discs able to grow in the former but not in the latter environment.

c. Growth tests in male and female hosts.

It is evident from the foregoing experiments that male and female environments are different as to their effect on the growth of the grafted organ discs. Decisive evidence for this is provided by the following experiments. The two

partners of the virilis leg disc pairs were transplanted, one into a male, the other into a female virilis host. Five such pairs were dissected three days, and eight pairs four days after the operation. In all pairs it was found that the discs in the female hosts were much larger than their partner discs in the male hosts, which had not grown at all. Figure 5 (*a*, *b*) illustrates this effect very clearly.

Similar results were obtained in another series of experiments (five pairs dissected three days after the operation), where leg discs pairs of virilis were transplanted into female and male melanogaster hosts.

In transplanting the two partners of virilis eye disc pairs into virilis male and female hosts (three pairs dissected three days after the operation), we again find the discs in the female hosts larger than their partners in the male hosts (Figures 6*a* and 6*b*). The eye discs in the male hosts had ceased to grow, being of the same size at the time of dissection as at the time of transplantation.

Finally, in a last series of this kind, the two partners of a pair of salivary glands of virilis larvae were transplanted, one into a male and the other into a female virilis host. From eight such pairs three were dissected in four days, two, seven days and three, eight days after the operation. Again, as in the case of the organ discs, it was found that the glands in the male hosts had not developed while their partners in the female hosts were all in an advanced stage of development.

d. Growth test in different host species.

The object of this group of experiments was to test for possible species differences between virilis and melanogaster hosts. To this end the two partner discs of a virilis leg pair were transplanted, one into a virilis male host and the other into a melanogaster male host. From six pairs available, two were dissected three days, two, four days and two, six days after the operation. The transplanted discs were found to be of the same size in both hosts in all pairs. There is evidently no difference between the melanogaster and virilis environment, as far as it affects the graft.

In a second series of experiments, comprised of seven pairs, which were dissected three days after the operation, the two virilis leg pair partners were transplanted into a melanogaster and a virilis female host. Being in a female environment, the discs in both hosts had, of course, grown; in one pair the discs were of the same size, in three pairs the discs in the melanogaster hosts were

PLATE II

FIGURE 6. Virilis eye disc pair three days after the operation. One partner disc (*a*) was transplanted into an adult virilis male host and the other partner disc (*b*) into an adult virilis female host. Note: disc in female host has become much larger than partner disc in male host, which has remained unchanged.

FIGURE 7. Virilis leg disc pair three days after the operation. One partner disc (*a*) was transplanted together with two ring glands into an adult virilis male host and the other partner disc (*b*) was transplanted together with two ring glands into an adult melanogaster male host. Note: disc (*b*) in the melanogaster host has become much larger than its partner (*a*) in the virilis host.

FIGURE 8. Virilis leg disc pair four days after the operation. One partner disc (*a*) was transplanted into an adult virilis male host together with four ring glands and its partner (*b*) into an adult virilis male host together with eight ring glands. Note: both discs have grown the same.

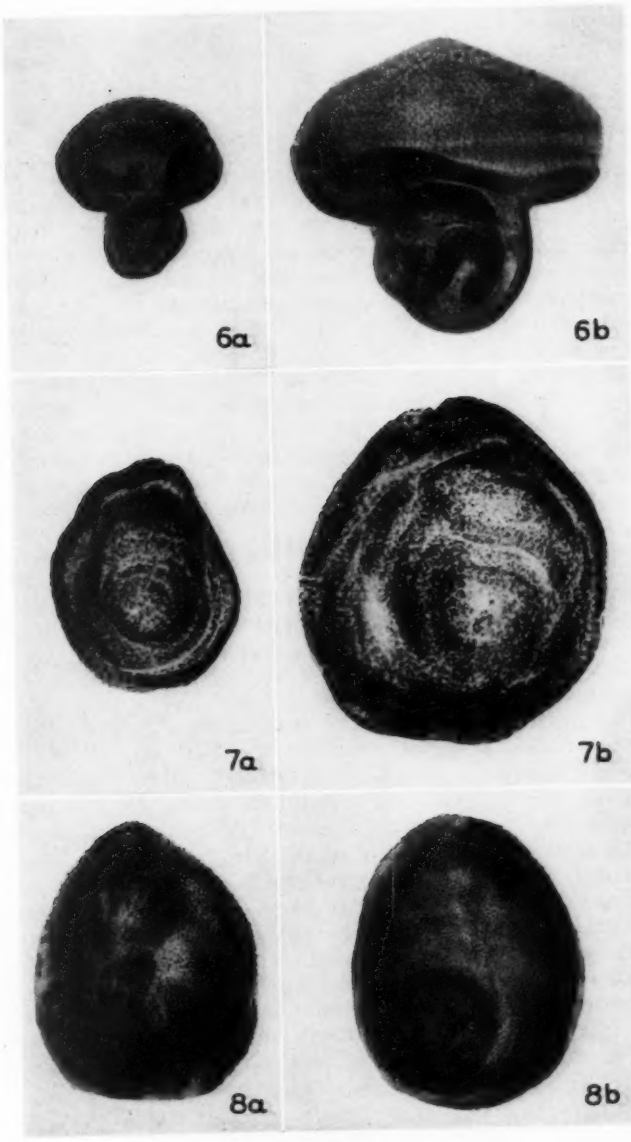

PLATE II

slightly larger and in the last three pairs they were somewhat larger in the virilis hosts. The fact that the discs may be larger in melanogaster female as well as in virilis female hosts indicates that there is no significant difference between the environment of both host species.

e. Growth test in male and female hosts in the presence of ring glands.

It has been shown before that organ discs grow larger in female hosts than in male hosts. The question now arises, how the growth of equal discs is affected when they are under the influence of the same number of ring glands in both environments. For this, two partner discs of a virilis leg pair were transplanted, one into a virilis male and the other into a virilis female host, while at the same time each of the two hosts in two of such pairs received five ring glands, and in two other pairs four ring glands from mature larvae. In dissecting the two pairs with five ring glands three days, and the two pairs with four ring glands four days after the operation, it was found that the discs in the female hosts of all pairs were larger than their partner discs in the male hosts. However, in contrast to the earlier experiments, where the discs were grafted alone, into male and female hosts, both discs in this experimental combination had grown. Moreover, the discs in the female hosts had become much larger in the presence of ring glands than discs which had grown in female hosts without ring glands.

f. Growth tests in different host species in the presence of ring glands.

If the experiments where virilis partner leg discs are transplanted into two hosts of different species (melanogaster male and virilis male) are repeated, but each host receives in addition two ring glands from mature larvae, then the results obtained are quite different. The transplanted organ discs are much larger in the melanogaster hosts (Figures 7*a* and 7*b*). This was observed in nine out of 12 pairs four of which were dissected three days, six four days and two six days after the operation. Only in three pairs left for four days in the host were the discs found to be alike in both hosts. Moreover in another pair, where one partner disc was transplanted into a virilis male host together with five ring glands, and the other partner disc into a melanogaster male host together with four ring glands, it was found again that the disc in the melanogaster host was much larger. Finally, in two additional pairs in which one partner disc was transplanted together with four ring glands, into a virilis male host, and the other partner into a melanogaster male host together with only two ring glands, it was again observed that, three days after the operation, the discs in the melanogaster hosts were much larger than their partners in the virilis hosts. Thus two ring glands in melanogaster hosts are able to induce more growth in the test organs than four ring glands in virilis hosts. Since in all these cases virilis ring glands were used as grafts we witness the peculiar fact that virilis ring glands are more effective in a foreign than in their own species environment.

II. The activating system

Experiments described in this section are designed to further the understanding of the ring gland action.

a. The effect of different numbers of ring glands on organ growth.

We have seen that organ discs transplanted into adult male hosts are able to grow only when under the influence of simultaneously transplanted ring glands. It remained to be shown, however, as to how many ring glands are actually needed to assure maximum growth of the organ disc. For this, eye discs of virilis larvae of equal size were transplanted from virilis male hosts while in addition, each of these hosts received a different number of ring glands from mature larvae. The results of the experiments are summarized in Table II,

TABLE II

Effect of different number of ring glands on eye growth.

Experiment	Number of ring glands	Transplant remains in hosts for three days		Transplant remains in hosts for four days	
		Number of cases	Size of eyes	Number of cases	Size of eyes
A	0	4	no growth	4	no growth
B	1	4	larger than A	4	larger than A
C	2	4	larger than B	4	larger than B
D	4	4	larger than C	4	larger than C
E	8	4	larger than C	4	larger than C

where it can be seen that two ring glands produce a greater growth effect in the test organs than one ring gland, but that the effect of four or eight ring glands is the same as that produced by only two ring glands. This evidence is further supported by experiments in which the two partners of single virilis leg pairs subjected to the influence of a different number of ring glands in virilis male hosts are compared. Table III summarizes the results obtained from six such

TABLE III

The effect of different numbers of ring glands on leg growth.

Number of ring glands compared	0 and 1	2 and 4	4 and 8	2 and 8	2 and 8	2 and 8
Days leg pairs remain in hosts	4	4	4	3	9	9
Size of leg pairs	larger in ring gland host	same in both hosts	same in both hosts	same in both hosts	same in both hosts	same in both hosts

pairs, showing again that the growth of the test organ is the same whether two, four or eight ring glands are present (Figures 8*a* and 8*b*). In summarizing the results, we must conclude that one ring gland is apparently unable to raise the hormone concentration in the adult male fly to a level high enough to assure maximal growth in the test organs. Yet the hormone concentration produced by two ring glands must have reached the level of saturation as far as the growth

of the test organ is concerned, since more than two ring glands have no greater effect than only two ring glands.

Evidence that the number of ring glands is also of importance for the time of imaginal differentiation of the test organ is provided by the following experiments. Melanogaster eye discs of equal size were transplanted into adult melanogaster female hosts, (*a*) alone, (*b*) together with two ring glands, (*c*) together with three ring glands, (*d*) together with four ring glands. All hosts were dissected eight days after the operation. It was found that in the hosts without ring glands the eye discs had grown, but were still white and showed no sign of imaginal differentiation (seven cases). In the hosts with two ring glands, reddish pigmented regions could be seen in the transplanted discs, showing that the ring gland had brought about pigment differentiation in the eye disc (six cases). The eyes in hosts with three ring glands (four cases) and in hosts with four ring glands (four cases), had developed to the same stage of pigmentation. In a further set of experiments melanogaster eye discs were transplanted alone into melanogaster female hosts but left for 16 days (two cases), 17 days (one case), and 22 days (one case), in the hosts before they were dissected. By this time pigmentation had also started in these eyes but was in a much less advanced stage of development. This is indicated by the slight yellow coloration, as contrasted with the reddish color, developed in eyes grown in hosts with ring glands. These experiments show that color development can take place in eye discs transplanted into female hosts without the support of ring glands, but that it is much less rapid than in female hosts in the presence of ring glands. The onset and degree of eye pigmentation in the presence of two, three or four ring glands in female hosts is about the same. Thus as in the experiments where the effect of different numbers of ring glands on the early growth of the eye discs was tested we find that for the later processes of differentiation also two ring glands produce the maximal effect.

b. The effect of ring glands of different age on organ growth.

Until now we have studied only the effects of mature ring glands, that is, of ring glands from larvae shortly before pupation. It remains to be seen, however, whether there is any difference in the effects produced by younger or older ring glands. Single pairs of virilis leg discs were thus transplanted, one partner alone and the other partner together with ring glands, into two virilis male hosts. The virilis ring glands used for each pair were of different age. In this way progressively younger ring glands were tested as to their effect on the growth of the organ discs. In two series of this kind, each host received three ring glands, in a third series, four ring glands. All pairs in the three separate series were dissected five days after the operation and the growth of the disc in each pair compared. The results of the experiments are summarized in Table IV, where it can be seen that ring glands of all ages, even when coming from larvae as young as five and a half days before pupation, i.e. young first instar larvae, are able to promote growth in the transplanted leg test disc. This proves that the ring gland can produce its growth hormone during the entire larval period of the animal. Whether there may be any interruption in the hormone production of the ring gland during this period, as the few negative cases might indicate (see Table IV), is not known, and needs further investigation.

c. Differences in hormone production of young and old ring glands.

The question as to the amount of hormone produced by young and older ring glands was tested in the following way: melanogaster leg discs of an average diameter of 13 units were divided into three lots. One set of legs was transplanted

TABLE IV

The effect of ring glands of various age on organ growth. In all cases the test organ remained for five days in the host. Positive indicates that the leg partner in the host with ring glands is larger than its partner in the host without ring glands; while negative indicates that both leg partners in the two hosts compared are of the same size.

Number of ring glands transplanted	Ring gland donor. Age in days before pupation	Ring gland donor, larval stage	Result
3	before pupation	3	positive
3	before pupation	3	positive
3	1	3	positive
3	1	3	positive
3	2	3	positive
3	2	3	positive
3	3	⅔	negative
3	3	⅔	negative
3	4	2	negative
3	4	2	positive
3	5	1	positive
3	before pupation	3	positive
3	before pupation	3	positive
3	1	3	negative
3	1	3	positive
3	3	3	positive
3	4	2	positive
3	4	2	negative
3	5	1	positive
3	5	1	positive
4	1	3	positive
4	1	3	positive
4	2	3	negative
4	2	3	positive
4	3	3	positive
4	4	2	positive
4	4	2	negative
4	5	1	positive
4	5	1	positive
4	5½	1	positive
4	5½	1	positive

into melanogaster females alone, the legs of the second set into melanogaster females each together with one melanogaster ring gland from an old larva shortly before pupation, and the legs of the third set into melanogaster females each together with one two-day younger melanogaster ring gland. Three days after the operation the hosts of these three groups were dissected and the transplanted

leg discs measured and compared. The legs in the hosts without ring glands (eight cases) had grown to an average diameter of 16 units. The legs in the second group with one young ring gland (five cases) were found to average 21 units in diameter and in the last group with one old ring gland, the average diameter of the legs (four cases) was 24 units. It was noticed, moreover, that the legs in the last group had begun to evaginate, which was not the case in the other two groups.

Now one may test, although in a somewhat different way, the amount of hormone produced. If it is true, as the above mentioned experiments indicate, that one young ring gland produces less hormone than one old ring gland, one might expect equal discs transplanted at the same time, and left long enough in the adult host, to be advanced further in their metamorphosis in the presence of old ring glands than in the presence of the same number of younger ring glands. In such an experiment we use the state of metamorphosis rather than differences in growth as an indicator for the hormone concentration. In order to elucidate this point, melanogaster eye discs of equal age were transplanted into adult hosts, some with four ring glands from larvae shortly before pupation and others with four one day younger ring glands. Eight days after the operation the hosts were dissected and the discs compared. The eyes in hosts with four ring glands had developed yellow-red pigment (four cases) while the eyes in hosts with four younger ring glands were much less advanced in their differentiation. Although they had grown extensively in the presence of the younger ring glands, they were still white, showing no trace of pigmentation (four cases). From these two groups of experiments we may thus conclude with reasonable certainty that young ring glands produce less hormone than old ring glands.

d. Species differences in ring glands.

Qualitatively the ring glands of virilis and melanogaster are the same. This has been shown many times in experiments where the action of virilis or melanogaster ring glands has been tested as to its effect on the growth and differentiation of melanogaster or virilis organ discs. The question whether there is any quantitative difference in the amount of hormone output during a given time between the ring glands of these two species is, however, not so clear. Since the ring gland of virilis is larger than that of melanogaster one might expect it to produce more hormone. If quantitative differences between melanogaster and virilis ring glands are present, they are at least not large, as the following experiment indicates. Equal melanogaster eye discs were transplanted into adult melanogaster female hosts together with two ring glands from a melanogaster larva shortly before pupation (three cases) and together with two virilis ring glands from larvae shortly before pupation (three cases). The dissection of these cases eight days after the operation showed that in the hosts with melanogaster ring glands, one eye disc had developed slight yellow pigment and two eye discs yellow-red pigment. In the hosts with two virilis ring glands, two eye discs had become slightly yellow and one eye disc yellow-red pigmented. We thus observe about the same amount of development under the influence of the same number of melanogaster or virilis ring glands, indicating that there is no difference in the quantity of hormone production between the ring glands of these two species tested.

e. The time of action of ring glands in adult hosts.

The question as to how long transplanted ring glands in adult hosts continue to produce hormone was tested as follows: virilis ring glands from larvae shortly before pupation were transplanted into adult virilis males. The glands were left in these hosts for a certain length of time, then dissected out and re-transplanted into a second adult host together with one partner disc of a leg pair. The other leg partner was transplanted alone into another virilis male host. After several days the pair of hosts was dissected, the growth of the leg discs compared and the ring gland grafts recovered. The recovered ring glands were now grafted for the third time into a virilis male host together with new test leg discs, the partners of which were again transplanted as the growth control into virilis male hosts alone. Several days later the pairs were dissected, the leg discs compared and the retransplantation procedure of the recovered ring glands using new test organs and new hosts repeated once or twice more. Since it was not easy to recover such a small organ as the ring gland from the body of the adult fly, two or four ring glands were usually transplanted together into one host. If one ring gland was lost in the dissection, the remaining ring glands could be used to continue the test. Table V shows the results of these experi-

TABLE V

The time of action of ring glands in adult hosts. (For explanation see text.) Positive indicates that the retransplanted ring gland has stimulated the growth of the test organ.

Experiment	A	B	C	D	E
Days ring gland remains in first host	6	6	10	10	22
Days ring gland retransplanted together with test organ remains in second host. Condition of test organ	5 positive	6 positive	6 positive	6 positive	6 positive
Days ring gland retransplanted together with test organ remains in third host. Condition of test organ	4 positive	5 positive		6 positive	
Days ring gland retransplanted together with test organ remains in fourth host. Condition of test organ		5 positive		4 negative	
Days ring gland retransplanted remains in fifth host. Condition of test organ		3 positive			

ments. Each of the five columns (A to E) represents one case of successive re-transplantation of the same original glands. Now, as it can be clearly seen from Table V, it was found that ring glands after being in adult hosts for 22 days, during which time they had been three times retransplanted and found to be active, were still active in a fourth transplantation (Table V B). Similarly, ring glands which were left for 22 days in one host before they were tested in a second host for their activity were still able to induce growth in the test

organ (Table V E). From these experiments we must conclude that ring glands transplanted into adult hosts secrete their hormone continuously for a long time.

III. The reacting system

It has to be realized that the various kinds of organ discs may differ as to their responsiveness towards the same hormone level. We may also expect differences in the responsiveness between old and young organ discs. Experiments which investigate these possibilities are presented in the following.

a. The differentiation capacity of different discs in adult hosts.

In comparing the first growth effect of such organs as eye and leg discs, one finds both very responsive to the hormone of the ring gland. Even in female hosts without ring glands, which must be considered the least favorable environment, the growth effect of both discs is considerable. Thus there seems to be no appreciable difference in the responsiveness between leg and eye discs. Yet when one compares the further development of these discs in the adult environment a marked difference between these organs becomes evident. One finds the leg discs able to differentiate in the adult host to imaginal completion under the influence of ring glands, but not the eye discs, which never continue their differentiation beyond the first stages of pigmentation. The leg discs in their final state of differentiation show typical imaginal characteristics; i.e. dark brown chitinized leg segments covered with chitinized hairs and bristles and with a blackish chitinized end claw on the distal tarsus segment. In the eye discs on the other hand, we find that the pigment is the only component which differentiates to an appreciable extent. There is however some doubt whether even pigmentation reaches its final imaginal stage. The development of hairs, bristles, lenses, or the darkening of the chitinous eye parts has never been observed in eye grafts. Although a more detailed histological examination of these partially developed eyes is still missing, there can be no doubt that differentiation is incomplete, since it would have been easy to detect chitinous structures in total mounts if they were present. It was found, moreover, that the anlage of the genital apparatus, when transplanted into adult hosts, is unable to differentiate at all, even in melanogaster female hosts in the presence of four ring glands, thus in an environment where the ring glands are most effective. Independent of the time these genital discs remain in the host, they never develop beyond a stage corresponding to the stage the discs would have reached in normal development at the time of puparium formation. In Table VI we have summarized a number of experiments in which different organ discs were transplanted together with ring glands into different adult hosts. Only such cases are recorded where the grafts were left for more than seven days in the host. We find, for example, that a melanogaster leg disc in a melanogaster female host in the presence of only one ring gland has already differentiated imaginal characters eight days after the operation, while a melanogaster eye disc in the same host in the presence of as many as four ring glands has developed only to the stage of pigment formation 19 days after the operation. Since about the same amount of pigment is present in melanogaster eyes which were left for eight days in female hosts together with two ring glands, it follows that the differentiation in the 19-day old eye has not progressed much beyond that observed in the eight-day old eye.

Thus in a melanogaster female host environment under the influence of two or more ring glands, the eye discs reach their limit of differentiation about ten days after the operation. As far as the genital discs are concerned, we find them to grow somewhat beyond their stage of transplantation. Their growth, however,

TABLE VI

The differentiation capacity of different discs in adult hosts.

Number of cases	Transplanted organ	Donor	Host	Number of transplanted ring glands	Days transplant remains in host	Result
1	♀ genital disc	viril.	viril. ♀	0	8	no clear change
1	♀ genital disc	mel.	mel. ♀	0	9	no clear change
2	♀ genital disc	viril.	viril. ♀	0	17	prepupal
2	♀ genital disc	mel.	mel. ♀	0	20	prepupal
2	eye	mel.	mel. ♀	0	16	light yellow spots; no hairs
1	eye	mel.	mel. ♀	0	17	light yellow spots; no hairs
1	eye	mel.	mel. ♀	0	22	light yellow spots; no hairs
3	eye	mel.	mel. ♀	1	9	red yellow spots; no hairs
1	eye	mel.	mel. ♀	1	16	reddish spots; no hairs
1	leg	mel.	mel. ♀	1	9	brownish hairs and chitin diff.
1	♀ genital disc	viril.	mel. ♀	4	7	little growth; prepupal
1	♀ genital disc	viril.	viril. ♀	4	8	little growth; prepupal
1	♀ genital disc	viril.	mel. ♀	4	9	little growth; prepupal
1	♀ genital disc	viril.	mel. ♂	4	9	little growth; prepupal
2	♀ genital disc	mel.	mel. ♀	4	9	little growth; prepupal
2	♀ genital disc	viril.	viril. ♂	2	10	little growth; prepupal
2	♀ genital disc	viril.	viril. ♂	4	10	little growth; prepupal
1	♀ genital disc	viril.	viril. ♀	4	10	little growth; prepupal
1	♀ genital disc	viril.	viril. ♂	4	13	little growth; prepupal
1	♀ genital disc	viril.	mel. ♂	4	13	little growth; prepupal
1	♀ genital disc	mel.	mel. ♀	4	14	little growth; prepupal
1	♀ genital disc	viril.	viril. ♀	4	17	little growth; prepupal
6	eye	mel.	mel. ♀	2	8	yellow red spots; no hairs
4	eye	mel.	mel. ♀	3	8	yellow red spots; no hairs
4	eye	mel.	mel. ♀	4	8	yellow red spots; no hairs
1	eye	mel.	mel. ♀	4	16	large reddish spots; no hairs
1	eye	mel.	mel. ♀	4	19	large reddish spots; no hairs
1	eye	mel.	mel. ♀	3	23	large reddish spots; no hairs
1	leg	viril.	viril. ♂	3	9	large; no hairs
4	leg	mel.	mel. ♀	3	9	brownish hairs and chitin diff.
1	leg	viril.	mel. ♀	4	9	brownish hairs and chitin diff.
2	leg	viril.	viril. ♀	3	10	hairs diff.; still white
1	leg	viril.	viril. ♀	3	10	brownish hairs and chitin diff.
1	leg	viril.	viril. ♂	3	12	hairs diff.; still white
2	leg	viril.	viril. ♀	3	13	brownish hairs and chitin diff.
1	leg	viril.	viril. ♂	3	14	brownish hairs and chitin diff.
3	leg	viril.	viril. ♂	3	16	brownish hairs and chitin diff.

is very much slower than that of leg or eye grafts, even in their early growth effect. They never surpass, as said before, a prepupal stage, although they may be as long as 17 days in a female environment under the influence of four ring glands.

TABLE VII

Comparison of the differentiation capacity of different discs from the same donor larvae transplanted together with ring glands into one adult host.

Experiment	Transplant	Donor	Host	Number of transplanted ring glands	Transplant remains in host	Condition of organ at dissection		
						Leg disc	Eye disc	Genital disc
A	leg eye disc	mel.	mel.	3	8	large, first white hairs	red yellow spots; no hairs	—
B	leg eye disc	mel.	mel.	3	8	large, first white hairs	red yellow spots; no hairs	—
C	leg eye disc	mel.	mel.	3	8	large, first white hairs	red yellow spots; no hairs	—
D	leg eye disc	viril.	mel. ♀	4	14	brownish hairs and chitin	large white; no hairs	—
E	leg eye genital disc	viril.	viril. ♀	3	8	large, no hairs	large white; no hairs	little growth; prepupal
F	leg eye genital disc	viril.	viril. ♀	3	11	large, no hairs	large white; no hairs	little growth; prepupal
G	leg eye genital disc	viril.	viril. ♀	3	11	large with white hairs	large white; no hairs	little growth; prepupal
H	leg eye genital disc	viril.	viril. ♀	3	11	large with white hairs	large white; no hairs	little growth; prepupal
I	leg eye genital disc	viril.	viril. ♀	3	11	brownish hairs and chitin	large white; no hairs	little growth; prepupal
J	leg eye genital disc	viril.	mel. ♀	4	9	large with white hairs	large white; no hairs	little growth; prepupal
K	leg eye genital disc	viril.	mel. ♀	4	13	large with white hairs	large white; no hairs	little growth; prepupal
L	leg eye genital disc	viril.	mel. ♀	4	13	large with white hairs	large white; no hairs	little growth; prepupal
M	leg eye genital disc	viril.	mel. ♀	4	13	large with white hairs	large, yellow spots, no hairs	little growth; prepupal
N	leg eye genital disc	viril.	mel. ♀	4	11	large with white hairs	large white; no hairs	little growth; prepupal
O	leg eye genital disc	viril.	mel. ♀	4	14	brownish hairs and chitin	large white; no hairs	little growth; prepupal

The capacity of the three imaginal discs tested to differentiate in adult hosts in the presence of ring gland thus decreases in the order leg, eye and genital disc. This can be demonstrated conclusively by a somewhat different experiment, as follows: a leg, eye and genital disc from the same donor larva were transplanted into a single host, together with ring glands. In this way all the three discs are under the influence of the same number of ring glands in the same host environment and can thus be compared more directly than in the previous experiments. Fifteen such cases are shown in Table VII, where it can be seen that the differentiation capacity of eye, leg and genital discs of the same animal differs markedly under the same hormonal conditions.

Before closing this section, one further point of importance should be mentioned. It has been found that there are also differences in the differentiation capacity in the various regions of the same organ disc. The clearest example of this phenomenon is provided in the differentiation of the leg disc. While it is true that leg discs are able to differentiate to imaginal completion in adult hosts, this statement must be modified somewhat, because it applies only to the distal leg disc portions. It is known that the larval leg disc not only includes the presumptive tissue of the actual adult leg, but also some material which gives rise to the ventral body wall in the nearest neighbourhood of the leg. This proximal portion of the leg disc never differentiates completely, while the distal leg portions consisting of femur, tibia and tarsus develop to imaginal completion. The differentiation capacity of the leg parts seems to increase in a proximal distal direction, since we find the tarsus segments always to be the first structures which become imaginal, and only if the discs are left longer in the host do we find tibia and femur completely differentiated. However, we have not been able to compel the proximal leg disc portions to become completely differentiated, although the discs have remained for a considerably longer time in the host than that needed for the complete differentiation of the distal leg parts. A very similar situation prevails in the eye discs, where we find that material which gives rise to pigment cells is able to express its differentiation tendencies, resulting in the formation of well-differentiated red pigment, while the material destined to form hairs or lenses is unable to differentiate to any great extent. Moreover, there seem to be regional differences in the eye for pigment formation also, for we observe most frequently that only certain eye regions are pigmented while others are still white. It seems possible that this last phenomenon might be correlated with the position of the graft in the host as well as with the arrangement of folding of the developing eye, which in turn may affect the oxygen supply in the different eye regions, and thus promote or inhibit, as the case may be, the oxidation of pigment.

b. Differences between young and old discs.

Young and old discs in the same hormonal environment differ as to their time of differentiation. This has been shown by the following experiment. Young and old leg or eye discs were transplanted simultaneously into single adult hosts together with one or more ring glands. The grafts were left in the host for not less than nine days; they were then dissected and compared. In all cases it was found that the older graft was further differentiated than the younger graft (see Table VIII).

TABLE VIII

Differences between young and old discs, transplanted together into one host.

Experiment	Transplant	Donor	Host	Number transplanted ring glands	Days transplant remains in host	Condition of organ at dissection	
						Old disc	Young disc
A	young and old eye	mel.	mel. ♀	1	9	large reddish spots; no hairs	large white; no hairs
B	young and old eye	mel.	mel. ♀	1	9	large reddish spots; no hairs	large white; no hairs
C	young and old leg	mel.	mel. ♀	1	9	large, with white hairs	large; no hairs
D	young and old leg	mel.	mel. ♀	1	9	large, with white hairs	large; no hairs
E	young and old leg	viril.	viril. ♀	3	10	large, with white hairs	large; no hairs
F	young and old leg	viril.	viril. ♀	3	10	large, with white hairs	large; no hairs
G	young and old leg	viril.	viril. ♀	3	10	brownish hairs and chitin	large; no hairs
H	young and old leg	viril.	viril. ♀	3	13	brownish hairs and chitin	large; no hairs
I	young and old leg	viril.	viril. ♀	3	13	brownish hairs and chitin	yellowish hairs and chitin

c. The responsiveness of organs of different species.

If one compares (Tables VI and VII) the final developmental condition of melanogaster eyes with virilis eyes which were left for the same length of time in an approximately equal adult environment, one observes that the melanogaster eyes have developed further than the virilis eye discs. This indicates a difference in the competence of virilis and melanogaster eyes to respond to the same hormonal conditions. The same indication is seen in another set of experiments where virilis leg discs were transplanted into virilis female hosts together with three ring glands (five cases) and melanogaster leg discs (four cases) into virilis females together with three ring glands. Eleven days after the operation two melanogaster legs had developed hairs which were, however, still white; the two other melanogaster legs were completely differentiated, showing yellow-brown chitinous structures and hairs. Three of the virilis legs were without any hairs, one had hairs but was white, and one was completely differentiated. Although these observations speak for the assumption that virilis organs respond with greater difficulty to the same hormonal environment, there is one further point to be taken into consideration. Virilis and melanogaster differ in their time of development. The larval as well as the pupal period of virilis is much longer than that of melanogaster. Therefore it is possible that in the above-mentioned experiments, leg discs of unequal age were compared, especially since no accurate record was made of the exact age of the donor discs. The observed difference in the time of differentiation between virilis and melanogaster organs may thus not really reflect species differences but rather age differences. Even if we assume that the organs in question were of the same age, this would mean only that they were alike in their chronological age but not in their physiological age. In the light of these considerations it becomes evident that it is rather difficult in experiments of this kind to be quite sure whether any discrepancies in the time of differentiation between discs of two species are caused by species-specific responses or age effects.

DISCUSSION

The present investigations have brought forward a number of pertinent facts concerning the relationship between hormone actions and tissue competence in

the development of Drosophila organ discs. It has been shown that the organ discs depend for their growth as well as for their imaginal differentiation upon the action of the ring gland which functions as a gland of internal secretion. As judged from their effect on test organs, young ring glands produce qualitatively the same hormone as ring glands of a mature larva. The quantity of hormone produced by young ring glands is presumably less than that produced by old ring glands. Equal ring glands differ as to their effect in hosts of different species and in the two sexes of the same species. The amount of organ growth during a given time and the speed at which differentiation proceeds depends upon the number of ring glands, i.e. on the amount of hormone available as well as on the competence of the organs to respond. Different organ discs as well as discs of different ages and different regions within the same organ disc differ as to their competence to respond. These facts reflect very clearly the highly relative nature of conditions which find their expression in the processes of growth and differentiation. They show that we cannot ascribe absolute values to either organ competence or hormone concentration but rather that we have to measure one in terms of the other.

We have now to consider in more detail certain aspects of the problem of hormone-controlled growth and differentiation which have arisen in the course of these investigations. For this it seems best to discuss separately the principal points in question, and after we have estimated their value to try to fit them into the framework of the general concept.

A. Relationship between hormone concentration and effective level.

Of particular interest is the observation that organ discs transplanted into adult female hosts are able to grow even in the absence of ring glands. Since we know that the growth of the transplanted organ is under the control of the ring gland hormone we might assume that female hosts, in contrast to male hosts, either produce or have stored some hormone. We know further that two ring glands have the same effect as four glands. In the presence of two or four ring glands, therefore, the environment of either female or male hosts must be considered saturated with hormone as far as the growth of the organ is concerned. We should thus expect the hormonal environment of female and male hosts to be the same, i.e. saturated when both hosts are supplied with five ring glands each. Consequently, we should also expect the growth response of identical organs grown in such a saturated male and female environment to be the same. This, however, is not the case, as the experiments show (see p. 42). The discs in the female environment grow much better than their partners in the male environment, although both were in a hormone-saturated environment. This suggests that the ring gland hormone does not act directly but, rather, indirectly by the intervention of some factors in the host. Limited by the lack of further knowledge on this point we might assume for the time being that the ring gland hormone establishes what might be called an "effective level" in the host, which in turn is responsible for the various reactions of the test organs. This assumption is supported by the fact that we observe similar differences in the reaction of the test organs under the influence of the same number of ring glands in different species. In these cases, too, a different growth effect is produced when the hormone concentration has saturated the environment.

For example, we find that two ring glands in melanogaster hosts have a greater effect on the growth of the test organ than four ring glands have in virilis hosts. However, there is a definite relationship between the hormone concentration and the effectiveness of this level. We find that a low hormone concentration produced by one ring gland is unable to raise the host level to its most effective state, while the hormone concentration produced by two ring glands already brings the level to its highest state of effectiveness. Although the effective level cannot be raised above a certain threshold even when higher hormone concentrations, i.e. more ring glands, are used, its peak effectiveness is nevertheless higher in females than in males and in melanogaster than in virilis. Yet there is no apparent difference in the effective level of the females in these two species when tested without ring glands. The difference between the species becomes evident only when their levels are elevated by the ring gland hormone. Whether the low effective level of female hosts is caused by the presence of a small amount of hormone is as yet still obscure, but of course possible. In any event, it seems unlikely that some hormone is stored, since in this case we would expect that the stored hormone would gradually decrease as the flies become older. The experiments show, however, that young and old flies are equally affected. Now, when one follows the thread of implications connecting these various points it becomes evident that one may obtain different effective host levels either by varying up to a certain point the hormone concentration, or by varying the host animals. For example, the lowest effective level prevails in female hosts without ring glands. The effective level is somewhat higher in male hosts with one ring gland. In melanogaster and virilis male hosts with two or more ring glands, the effective level is lower than in the virilis female hosts with two or more glands, while in a melanogaster female with two or more ring glands the effective level is highest. If in the following we speak of hormone concentration, it should be understood that we always refer to a host level of a certain effectiveness, produced by a definite concentration of ring gland hormone in a definite host.

It is characteristic that organ discs are unable to grow in adult male hosts without the support of ring glands. The male host environment was thus considered neutral. Now we know only that the adult male environment is neutral as far as the larval discs are concerned. Whether pupal organs which are presumably much more responsive than larval organs are also unable to develop in male hosts is not known so far. Actually it would be very difficult to prove that such an environment is neutral in an absolute sense, i.e. for all larval as well as all pupal tissues. If we should find, for example, that pupal discs, but not larval discs, would develop in adult male hosts and from this conclude that the pupal discs have attained the capacity of independent development, this conclusion could well be erroneous. We must take into consideration that the effective level in the male hosts, although too low for the growth of the larval discs, might well be high enough to assure the development of the highly responsive pupal discs. This argumentation brings us directly to one further aspect of the problem. In an earlier paper (Bodenstein, 1939a) it was shown that eye discs of young pupae continue their development when transplanted into larvae the anterior parts of which were cut off by means of a ligature. From these experiments the conclusion was drawn that pupal eye discs, which already had been stimulated by the differentiation-promoting hormone, are able to

develop independently in an environment lacking the differentiation stimulus. At the time these experiments were performed we did not know that the ring gland is the source of the hormone which promotes differentiation, nor that this hormone is produced in younger larval stages. The larval host therefore was expected to contain no differentiation hormone. Although the source of the hormone supply in these earlier experiments was cut off by the ligature, and thus no hormone coming from the ring gland could have reached the transplant, it is highly probable that enough hormone was left in the rear part to account for the continued development of the transplanted organ. Since we must assume that even a very low hormone concentration is sufficient to affect the very responsive older eye discs, this experiment does not prove the independent development of the pupal eye. Ephrussi (1943) has recently performed a similar experiment. He transplanted eye discs from mature larvae into the abdomen of young larvae and observed that these discs developed synchronously with the host organs. However, in another series of experiments where he transplanted eye discs of one-day old pupae into young larvae he found the transplanted eyes to develop heterochronously. In these cases the transplanted pupal eye had already formed red pigment while the hosts were still in their larval stage. These experiments also do not prove the independence of eye development, for the hormone concentration in the young larvae, although not high enough for the differentiation of the larval host organs, might have been sufficient for the differentiation of the pupal eye. In the light of these considerations, it is very difficult indeed to be sure whether one is dealing with dependent or independent development. Again we are confronted with the fact that development is not the reflection of absolute conditions, but is highly relative indeed; it is the expression of a very delicate balance between the activating and reacting systems involved.

B. The effective level and tissue competence.

It takes about eight days for a leg disc to differentiate to imaginal completion in a very effective female environment obtained by a hormone concentration produced by two or more ring glands, while in normal development in the presence of only one ring gland the leg disc completes its differentiation in four days. This shows that the effective level in the normal pupal environment must be much higher than that of the most effective adult environment. This low level in the adult environment is very fortunate for the understanding of the responsive capacity of the test organs, since it has brought to light real differences in the responsiveness of different test organs and of different regions within identical organs. For example, if we compare different discs as to their capacity to differentiate, we find in the most effective adult environment only the distal parts of leg discs are able to complete their imaginal differentiation, while under the same conditions, eye discs differentiate only partially and genital discs not at all. These differences in the responsive capacity of the different discs are not detectable if we grow them in a pupal environment under the influence of a very effective level. For, if we transplant legs, eyes and genital discs into larvae shortly before pupation, all these discs become mature in complete synchrony with the host organs and there seems to be no difference between them as far as their responsiveness is concerned. We have demonstrated that young and old

discs grown in a highly effective adult environment differ in their time of onset of differentiation. The young leg discs begin and complete their differentiation considerably later than older leg discs. When finally even the young discs have attained imaginal character they are of approximately the same size as the older discs. In other words, the young discs grow to a certain size before their differentiation leading to imaginal completion begins. This seems in disagreement with the results of earlier experiments (Bodenstein, 1939b) where it was found that young eye discs transplanted into older larval hosts differentiated prematurely, that is, before they had reached their full larval size, and as a consequence were finally smaller than normal eyes. When we recall that the effective pupal level is much higher than even the most effective level in an adult environment, we realize how we can explain the observed discrepancies between the results of our earlier and present experiments. Obviously, the pupal level is high enough to induce premature differentiation in the young organ while the adult level is able only to promote growth in the young organ. Only after the young disc in the adult environment has grown to a certain stage and has thereby become more readily responsive is the low effective adult level able to induce differentiation also into the young disc. Experiments in which the responsiveness of young and old salivary glands was tested (Bodenstein, 1943a in press) yield the same results. These experiments show that differentiation takes place only when both organ-responsiveness and effective level together attain a sufficient value. The difference between the responsive capacity of young and old organ discs is also clearly demonstrated by experiments (Bodenstein, 1939a and b) in which very young eye discs were transplanted into larvae shortly before pupation. In these cases the very young eye discs were only partly differentiated at the time the host emerged, although they had been under the influence of the very effective pupal level. This shows that even the very effective pupal environment is unable to bring about complete differentiation in test organs which are very young and hence possess a very low responsive value.

If we list the different organ discs as to their capacity to differentiate in the most effective adult environment, we find them arranged in the following order: legs, larval salivary glands, eyes, adult salivary glands and genital discs. Under the influence of the same effective adult level we thus find that the value for the differentiation response is highest in the leg disc and lowest in the genital and adult salivary gland discs, while the values for the other discs tested fall between these extremes. However it seems that the larval skin is more readily responsive than all the organ discs, as the following experiments by Hadorn and Neel (1938) indicate. The authors transplanted ring glands into young larvae of the early third instar and found that under the influence of the ring gland grafts puparium formation took place prematurely, yet these prepupae failed to develop further. This indicates that the larval skin is very responsive indeed, since it responded to the increased hormone level with puparium formation, before the organ discs were able to respond and hence failed in their differentiation.

Viewing the specific results of the investigations we conceive the following general picture of the mode of action of the ring gland in the development of Drosophila. The larval ring gland of Drosophila is an organ of internal secretion which produces its hormone during the entire larval period. This hormone controls the growth of organ discs during larval life. In the course of larval

development the ring gland becomes larger and produces more hormone, while at the same time the responsiveness of the organ discs increases as they grow older. When the hormone concentration and the responsiveness of the organ discs have reached a certain value, the ring gland hormone controls imaginal differentiation also. The evagination of the organ discs is the first indication that they have reached a differentiation phase. In normal development this stage is reached at the time of pupation. Pupation is thus nothing more than the first step in the process of differentiation. The kind of organ response, i.e. whether the organ discs respond with growth or differentiation to the ring gland hormone depends upon a definite relationship between hormone concentration and organ responsiveness. It is very probable that the ring gland hormone has no direct effect on the reacting organ systems, but that it rather acts indirectly through the intervention of some as yet unknown mechanism. If these conclusions deduced from experimental results are correct, it should follow that extirpation of the ring gland in the larval stage should prevent the growth of the organ anlagen. This experiment, technically not possible in Drosophila, has actually been performed by Burtt (1938) on Calliphora larvae, with the result that the growth of the organ disc was arrested in larvae which had their ring glands removed. These experiments then provide further evidence that the ring gland hormone controls not only differentiation but also the processes of organ growth during the larval period. The general interpretation of the problem under discussion is in contrast to Hadorn's view; he maintained that only ring glands from mature larvae produce hormone and that this hormone controls solely the processes of puparium formation, but has no effect on the growth or differentiation of the organ discs. On the basis of our experimental evidence, Hadorn's conception seems to be no longer tenable.

Summary

A variety of organ discs of Drosophila was transplanted together with or without ring glands into the body cavity of adult flies and their developmental behavior in their new surroundings studied. The specific results of these investigations are briefly summarized as follows:

1. Organ discs transplanted into adult male hosts cease to develop but remain alive presumably indefinitely. The transplanted discs do not lose their developmental potencies, although development may be arrested for a long time.

2. Organ discs transplanted into adult male hosts will grow and finally differentiate to imaginal completion when under the influence of simultaneously transplanted ring glands.

3. Organ discs transplanted into adult female hosts continue their development at a very slow rate even in the absence of ring glands.

4. There is no difference in the organic environment of different species as far as the development of test organs is concerned. If however, different host species are provided with the same number of ring glands it is found that the ring glands have a greater effect on the development of the test organs in melanogaster than in virilis hosts.

5. Ring glands of all larval ages, even from larvae only 12 hours old, are able to induce growth in the transplanted test organ.

6. The amount of hormone produced by young larval ring glands is less than that produced during the same time interval by ring glands of mature larvae.

7. Different organ discs differ as to their capacity to differentiate in adult hosts under the influence of ring glands.

8. Different regions within the same organ disc also differ as to their differentiation capacity.

9. Under the same hormonal environment it takes the young organ discs a considerably longer time to complete differentiation than it takes the old organ discs.

10. The ring gland hormone, apparently, does not affect the reacting organ directly, but acts rather through the intervention of some as yet unknown factors in the host.

11. The kind of organ response, that is, whether the organ disc responds with growth or differentiation to the ring gland hormone depends upon the relationship between hormone concentration and organ responsiveness.

12. The problem of growth and differentiation in the development of Drosophila is discussed. It is pointed out that development is not the reflection of absolute conditions but that it is highly relative indeed; it is the expression of a very delicate balance between the activating and reacting systems involved.

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THE LIFE-HISTORY OF *PHYLLODISTOMUM SOLIDUM* RANKIN, 1937, WITH OBSERVATIONS ON THE MORPHOLOGY, DEVELOPMENT AND TAXONOMY OF THE GORGODERINAE (TREMATODA) [1]

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INTRODUCTION

Digenetic trematodes are internal flatworm parasites which occur in all classes of vertebrates. The life-cycles of these parasites involve two or more hosts of which one, the first intermediate, harbors the parasitic asexual stages and eventually liberates a transfer stage which is typically a free-swimming cercaria; the definitive host is usually a vertebrate which harbors the parasitic sexual stages of the worm. The cyclic transfer from one host to another has been variously modified by interpolations of additional intermediate hosts, and by complications in the methods of infection of the first intermediate hosts.

Trematodes belonging to the family Gorgoderidae Looss, 1901 are characterized by having their bodies generally divided into two regions: a narrower, mobile preacetabular part and a broader, sluggish postacetabular region. The cuticula is usually smooth. The intestine may be simple to ramified. The genital pore is median, preacetabular and behind the bifurcation of the gut caeca. The testes, two to nine in number, are intercaecal or extracaecal, usually oblique, rarely opposite. The ovary is usually pretesticular. The vitellaria are paired and postacetabular. The uterus is much coiled postacetabularly. The eggs are relatively small, numerous, without filaments, and usually fully embryonated. Adults are found in the excretory ducts and urinary bladders of fishes, amphibians and reptiles, and in the body and pericardial cavities of marine elasmobranchs.

The Gorgoderidae consists of two subfamilies with contrasting characters as follows:

Gorgoderinae Looss, 1899	Anaporrhutinae Looss, 1901
1. No pharynx.	1. Well-developed muscular pharynx.
2. Laurer's canal present.	2. No Laurer's canal.
3. No seminal receptacle.	3. Prominent seminal receptacle.
4. Parasites in urinary bladders and ducts of fishes, amphibians and reptiles.	4. Parasites in pericardial and body cavities of marine elasmobranchs and urinary bladders of marine turtles.

The subfamily Gorgoderinae, at present, contains the following genera: *Gorgodera* Looss, 1899; *Gorgoderina* Looss, 1902; *Phyllodistomum* Braun, 1899; *Xystretum* Linton, 1910; *Macia* Travassos, 1922. The genus *Phyllodistomum*

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has synonymy as follows: *Spathidium* Looss, 1899; *Catoptroides* Odhner, 1902; *Microlecithus* Ozaki, 1926; *Dendrorchis* Travassos, 1926.

The subfamily Anaporrhutinae, according to Nagaty (1930), contains the following genera: *Anaporrhutum* Ofenheim, 1900; *Probolitrema* Looss, 1901; *Plesiochorus* Looss, 1901; *Petalodistomum* Johnston, 1912; *Staphylorchis* Travassos, 1920.

Generic diagnosis of *Phyllodistomum* Braun: Body usually spatulate. Ventral sucker usually larger than oral sucker. Intestinal crura separate posteriorly. Genital atrium usually present. Testes two, smooth to deeply lobed, usually oblique, the one on ovarian side being more posterior. Anterior testis may be cephalad of ovary. Vas deferens long, seminal vesicle conspicuous, pars prostatica and ductus ejaculatorius generally short and inconspicuous. Ovary entire or lobed, usually posterior or lateral, rarely anterior to vitellaria. Oviduct relatively long, arising from dorsum of ovary. Fertilization space usually evident. Laurer's canal usually paralleling vitelline duct on side opposite to ovary and opening to exterior. Mehlis' gland present but indistinct. Metraterm large and distinct. Vitellaria compact or lobed; common vitelline duct very short. Eggs in metraterm embryonated. Excretory vesicle sac-like, elongate, its pore usually subterminal.

Gorgoderid trematodes are usually found in the exretory systems of their hosts; however, von Olsson (1876) described *Distoma conostomum* from the esophagus and gills of *Coregonus maraena*. Nybelin (1926) stated that the worms probably had emigrated from the bladder upon the death of the host, and that he always found this species of trematode in the urinary bladder. Van Cleave and Mueller (1934) have likewise reported ectoptic *Phyllodistomum superbum* from the gut of *Esox lucius*, *Percina caprodes zebra* and *Percopsis omnisco-maycus* from North America. Both Looss (1899) and Odhner (1902), upon the basis of morphological similarity with other known phyllodistomes, agreed that von Olsson's *Distoma conostomum* is a species of *Phyllodistomum*.

There have been more than 40 seemingly valid species described in this genus.

According to Nybelin (1926) the first report of an adult phyllodistome is probably that of Fabricus (1780) who described *Fasciola umblae* from the "kidney" of *Salmo alpinus* ("*in sanguine dorsali salmoni alpine*"). Later Fabricus (1794) redescribed and figured this same form. Rudolphi (1819) renamed it *Distoma seriale*, expressing the opinion that "*sanguine dorsali*" is probably to be interpreted as the kidney.

In the year 1816 v. Olfers described from *Esox lucius* a bladder fluke which he called *Distomum folium*. Many of the distomes found by subsequent workers in the bladders and urinary ducts of fishes and amphibians have been referred to this species. This fact has created the extremely difficult taxonomic problem of attempting, with meager data, the separation and identification of valid species.

Braun (1899) erected the genus *Phyllodistomum* with *Dist. folium* Olfers, 1816, as type, and included in the genus: *Dist. cygnoides* (Zed.) Looss, *D. cymbiforme* Rudolphi and *D. patellare* Sturges. Later in the same year Looss erected the genus *Spathidium* with *D. folium* Olfers as type. By the laws of taxonomic priority, *Spathidium* has been suppressed as a synonym of *Phyllodistomum*.

Zschokke (1884) found *Distomum folium* in *Cottus gobio*, *Thymallus vulgaris*, *Trutta variabilis* and *Salmo umbla*, but not in *Esox lucius*. Braun (1892) found

Distomum folium in *Esox lucius* at Königsberg. His account indicates that Zschokke had confused: the ovaries (2) with the vitellaria, the oviducts with the vitelline ducts; he failed to see the ovary; the shell gland was confused with the vitelline reservoir and the vitellaria were eggs in the uterus. Looss (1894) reported *Distomum folium* from the urinary ducts of *Acerina cernua*. Sinitsin (1905) studied fishes from Warsaw ponds and identified *Phyllodistomum folium* in *Carassius vulgaris, Barbus vulgaris, Gobio fluviatilis, Leuciscus rutilus, Scardinius erythrophthalmus, Squalius cephalus, Idus melanotus, Aspius rapax, Abramis brama* and *Blicca björkna*. Finally, Zandt (1924) found *Phyllodistomum folium* in *Leuciscus leuciscus* and *Leuciscus rutilus* from Lake Constance.

Looss (1901) described *Phyllodistomum acceptum* from *Crenilabrus pavo* and *C. griseus*. Odhner (1902) described *Phyllodistomum unicum* from *Serranus sp., Phyllodistomum linguale* from *Gymnarchus niloticus, P. spatula* from *Bagrus docmac* and *B. bayad*, and *P. spatulaeforme* from *Malapterurus electricus*. He hesitated in placing the last two phyllodistomes in the genus because they had symmetrically placed testes and sharply separated anterior and posterior body regions. It was due to this hesitation that Odhner in Looss (1902) erected the questionable genus *Catoptroides* with *P. spatula* Odhner as type.

Osborn (1903) described *P. americanum* from North American *Amblystoma tigrinum*. Since that time, descriptions of phyllodistome species have been generally complete enough to reduce taxonomic difficulties.

Two complete and several incomplete life-histories have been reported for members of this subfamily. Incomplete cycles were determined by Sinitsin (1905) for three species of *Gorgodera* and *Gorgoderina vitelliloba*. Krull (1935) experimentally proved the life-cycle of *Gorgodera amplicava*. Rankin (1939) determined the life-history of *Gorgoderina attenuata*, the first for any North American species of that genus. Except for abstracts by Crawford (1939, 1940) on the life-history of *Phyllodistomum americanum*, and Goodchild (1940) on the life-history of *P. solidum* there have been no life-histories reported for North American phyllodistomes.

Several European workers have postulated, upon morphological similarity and slight experimental evidence, certain relationships between cercariae or metacercariae and sexually mature phyllodistomes. Nybelin (1926) summarized and amplified these speculations concerning European phyllodistome life-histories. Looss (1894), Lühe (1909) and Odhner (1911) proposed that *Cercaria duplicata* v. Baer which develops in *Anodontites cygnea, Anodontites anatina* and *A. cygnea ventricosa* is the larva of *Phyllodistomum folium* (Olfers). Nybelin (1926), on the other hand, stated his belief that *C. duplicata*, as at present defined, consists of three separate cercariae: (1) *C. duplicata* Wagener, 1851 (Nybelin stated that this larva must be removed from the phyllodistome cercariae because it possesses a pharynx and has a Y-shaped excretory bladder); (2) *C. duplicata* Pagenstecher, 1857 (also accredited with a pharynx, which Nybelin regards as an error in observation)—Nybelin was unable to link this cercaria with any known adult phyllodistome; (3) *C. duplicata* Reuss, 1903 (= *C. duplicata* v. Baer according to Nybelin). In extensive feeding experiments of metacercariae from *C. duplicata*, using 16 different species of fish, several of which are natural hosts for *Phyllodistomum folium*, Reuss was unable to obtain any sexually mature distomes. He found metacercarial excystment only in two species, *Cyprinus*

carpio and *Tinca vulgaris*, but there was no progressive development; thus he was unable to determine the species of the adult phyllodistome. Nybelin postulated, upon the basis of the sucker ratio, the position of the genital primordium and the position of the genital pore, that *C. duplicata* of Reuss is the larva of *Phyllodistomum elongatum;* this hypothesis seems untenable because Reuss was unable to find metacercarial excystment in *Abramis brama* which Nybelin listed as a normal host for the sexually mature *P. elongatum*.

Nybelin believed that the short-tailed cercaria described by Sinitsin (1901) as the larva of *Phyllodistomum folium* is a true phyllodistome cercaria, but is not that of *P. folium* because the cercaria had an acetabulum smaller than the oral sucker; the smallest adult *P. folium* which Nybelin observed (0.45 mm. long), had an acetabulum larger than the oral sucker. Nybelin stated that this short-tailed cercaria may represent the larva of *P. elongatum*, the other known bladder fluke of cyprinids. This assumption is inconsistent, however, because in the same paper he had already proposed the *C. duplicata* of Reuss to be the larva of *P. elongatum*, "Die einzige Larvenform für welche eine Vermutung der Artzugehörigkeit einigermassen berechtigt erscheint, ist die von Reuss näher behandelte, welche durch ihre etwa gleichgrossen Saugnäpfen, durch die Anlage des vorderen Hodens schräg hinter der Anlage des Germariums und vor allen durch die dicht vor dem Bauchsaugnapf gelegene Anlage des Genitalporus sehr an *Ph. elongatum* erinnert." This seeming contradiction Nybelin avoided by a second assumption as to the fate of the cercaria described by Reuss, "Es ist also auch in diesem Falle nicht möglich, etwas bestimmtes zu sagen; es wäre sogar denkbar, dass sich Reuss' Cercarie zu *Ph. pseudofolium* entwickeln könnte." However, the validity of this species has been questioned; Lewis (1935) threw *P. pseudofolium* into synonymy with *P. folium*.

Odhner (1911) stated that the stumpy-tailed cercaria of Sinitsin (1901) is the larva of *P. macrocotyle*. However, Nybelin (1926), Zandt (1924) and Lewis (1935) agreed that *P. macrocotyle* is synonymous with *P. folium*. Odhner (1911) believed that the marine "Rattenkönigcercaria" *Cercaria clausii* when eaten by the fish *Chrysophrys aurata* develops into *Phyllodistomum acceptum*. This belief was accepted by Steelman (1938) who accordingly suppressed *P. acceptum* as a synonym of *Phyllodistomum clausii*.[3] Nybelin summarized these life-history studies and speculations in his statement, "Es ist also gegenwärtig für keine *Phyllodistomum*-Art möglich, die zugehörige Cercarienform sicher anzugeben; die postembryonale Entwicklungsgeschichte der einzelnen Arten dieser Gattung muss vielmehr ganz von neuem, und zwar am bestem auf experimentellem Wege studiert werden." This is the only way reliable results are obtainable; and furthermore, life-history studies based upon controlled experimentation will be invaluable for clarification of the chaotic taxonomic status of the sexually mature phyllodistomes.

The first phyllodistome life-history to be experimentally completed in the laboratory was reported by Goodchild (1940) for *Phyllodistomum solidum* Rankin, 1937 which consisted of the following stages: sexually mature distomes in the urinary bladder of *Desmognathus fuscus fuscus*, sporocysts and young cercariae in *Pisidium abditum*, and metacercariae in naiads of several species of Odonata.

[3] Cable [1942. *Jour. Parasit.* 28 (6 suppl.)] proposes that *Cercaria clausii* is the larva of a fish trematode belonging to the family Lepocreadiidae or the family Gyliauchenidae.

In the present paper, the various stages of the parasite are described and experiments proving the life-cycle are cited.

The author wishes to acknowledge the encouragement and helpful criticism given by Professor H. W. Stunkard during both the progress of the experimental portions of the study and the preparation of the manuscript.

Materials and Methods

A. Bivalves and asexual stages.

The fresh-water sphaeriid hosts of gorgoderid trematode asexual larval stages are grouped into the three genera: *Sphaerium*, *Musculium* and *Pisidium*. *Pisidium abditum*, host of *C. Phyllodistomum solidum*, were collected from Barrett Pond near Cold Spring on Hudson, N. Y., in October and November 1937, and during the summers of 1938, 1939 and 1940. The clams were collected by careful examination of small quantities of bottom in regions where they were abundant. In this way, two collectors were able to take about 300 clams in one day. In the laboratory they were separated into lots of about 25 to 30 each and placed with a small amount of pond debris into finger-bowls. They were then set in running water to maintain a lower temperature. Every morning each container was examined for cercariae with the aid of a 3X lens; this method proved adequate for locating the cercariae, but as a final check, each bowl was examined under a 17X binocular microscope.

When cercariae were found in any dish, the clams in that bowl were then isolated to obtain the infected ones. All infected bivalves were placed in individual containers; this separation reduced the death rate because infected clams are less tolerant of conditions of crowding, increased temperature, starvation and fungal infections than uninfected molluscs.

It was extremely difficult to keep the clams alive in the laboratory. The water in which they lived was changed at least once a day. Culture water containing various species of green algae and diatoms was introduced in small amounts at frequent intervals. Nevertheless, the mortality during the period of adjustment to laboratory conditions was discouragingly high. When kept in running city water the results were more encouraging. Finally, an aquarium was set up which approximated the conditions in nature as much as possible. The pond debris which served as the bottom was sloped to one end of the tank. The depth of water was approximately two inches in the deepest part. At the other end, moss was introduced and a fine stream of water was allowed to trickle over it. It was possible in this way to have laboratory-raised specimens for miracidial infection experiments.

Infected bivalves were teased apart to obtain mature living sporocysts for study. Mature sporocysts were also fixed in warm (65°) Bouin's fixative and stained with paracarmine and Delafield's haematoxylin.

B. Odonatan naiads and metacercariae.

The damsel-fly naiads which were used as the second intermediate hosts were both grown from eggs in the laboratory, and collected from a small pond in Van Cortlandt Park, New York City. This pond was free of *Pisidium sp.*, so the naiads were the equivalent of laboratory-raised specimens. As a control,

25 per cent of the damsel-flies were dissected under the binocular microscope; they were all free of any helminth metacercarial infection. In addition, the naiads which were to be used as hosts were isolated in the laboratory for two weeks, after which time they were starved for periods of from three to seven days. Starvation made them so transparent that examination by transmitted or reflected light would disclose immediately any metacercariae. This period of starvation served the additional function of causing the naiad to capture and to devour eagerly any cercariae offered.

The cercariae were removed singly by micropipettes and introduced into a finger-bowl with a starved damsel-fly naiad. As soon as the nymph had seized a cercaria it was placed under the microscope and the penetration, wandering and encystment of the cercaria were watched.

Encysted and mechanically excysted metacercariae were studied alive, and also after fixation in Bouin's fluid and staining with Delafield's haematoxylin, Ehrlich's haematoxylin and paracarmine. Serial sections of experimentally infected odonatan naiads were also studied.

C. Desmognathus fuscus fuscus and adult worms.

The salamanders which were used in the experiments were captured as young adults. They were kept in the laboratory for periods of from 12 to 17 months before being used. During this time they were isolated at monthly intervals for periods of from three to seven days in a small quantity of water in a finger-bowl. The water in which they were living was examined about four times a day for free-swimming miracidia. Since, in agreement with Rankin (1939), it was found that miracidia will live under these conditions for as long as 24 hours, any miracidia shed would be readily found. Salamanders kept under identical conditions as controls, when dissected, were always found to be parasite free.

Adult worms were studied alive and after fixation. Bouin's fluid and saturated mercuric chloride solution with about 5 per cent acetic acid added were used as fixatives. Whole mounts were stained with paracarmine. Serial sections were stained with Ehrlich's haematoxylin and Mallory's triple connective-tissue stain.

Experiments

1. Experiments with first intermediate hosts:

Infected *Desmognathus fuscus fuscus* were isolated in finger-bowls in small quantities of water. The miracidia, which were given off in great numbers, were picked up in a dropper and introduced into a finger-bowl containing actively crawling laboratory-raised bivalves which were about 2 mm. long and approximately six months old. The miracidia moved about in more or less direct courses. They were not attracted to the bivalves; they would swim within less than 0.1 mm. of the clams and not change their courses. Miracidia were observed being drawn into the mantle cavity of the bivalve by way of the incurrent mantle cleft. The miracidium of *Gorgoderina attenuata*, according to Rankin (1939), enters its bivalve host similarly.

A more successful method of infection of molluscs was to introduce an infected *Desmognathus* into an aquarium with clams enclosed in small wire baskets. The salamander and bivalves were then allowed to remain together for as long as one week. It was possible, in this way, to obtain 50 to 70 per cent infections in the clams.

2. Experiments with second intermediate hosts:

Damsel-fly naiads were offered cercariae within four hours of emergence of the latter from bivalves. The violent action of the cercariae attracted the attention of the insect nymph. The labium of the naiad was projected out to grasp the cercaria which was then pulled within reach of the mandibles. The enlarged portion of the tail, because of its turgid spherical cells, prevented damage to the distome in the chewing process. However, it was found that such protection is not absolute, because about 15 per cent of the cercariae eaten failed to establish themselves in the insect host. Some of the failure may be due to imperfect cercariae, but in other cases, lacerated and partly digested cercariae have been found in sections of the gut.

The insect nymph was able to engulf the cercaria completely in approximately ten seconds. The tail collapsed and was digested, while the young distome penetrated the intestinal wall. Usually only one minute elapsed from the time the damsel-fly naiad took the cercaria until the young distome had penetrated the crop wall. In one instance, the point of penetration was in the prothorax; the worm migrated posteriad to the anterior edge of the metathorax, then turned and wandered to the anterior edge of the mesothorax where encystment finally took place. Usually the cercaria encysted, within four minutes of penetration, in the segment in which it pierced the crop.

The act of penetration caused apparent discomfort to the insect. With one cercaria the reactions were as follows: within 15 seconds after the act of swallowing, the naiad rubbed the legs together and also rubbed the body with the legs; then it started nervous wriggling movements, followed by short random dashes through the water. If several cercariae were taken within ten seconds of each other multiple penetrations occurred. The initial symptoms of the insect were then intensified, but otherwise similar to those described above. However, after about one minute the naiad lost its equilibrium and went into tetanus. A damsel-fly thus treated would not take additional cercariae for at least 30 minutes.

Similar behavior of odonatan naiads during penetration of gorgoderid cercariae has been reported by Sinitsin (1905) and Krull (1935).

The forming metacercarial cyst wall was recognizable as a delicate flexible covering within 15 minutes after cercarial penetration. The anterior end of the worm could easily push the wall out of shape. After cystogenous material, which was emitted through the excretory pore, had been deposited in one region of the forming cyst, the cercarial body was then thrown into a figure-8 shape which brought the oral sucker into contact with the extruded cystogenous material. The oral sucker and edge of the mouth opening then pushed the material into a smooth layer on the inside of the growing cyst wall. Meanwhile, the posterior end of the worm had been depositing cystogenous material elsewhere. The formation of the complete cyst took approximately 18 minutes; the activity then gradually diminished, and the young worm was practically quiescent one hour after penetration. The metacercaria changed position in the cyst, as indicated by drawings made over a period of several days.

Metacercariae were usually located in the thorax of the naiads; they have been found as far posteriorly as the second abdominal segment, and as far anteriorly as the dorsum of the head.

Various odonatan species have been tried as second intermediate hosts: *Ischnura verticalis*, and *Argia sp.*, were satisfactory; *Enallagma sp.*, and *Libellula*

sp., were less satisfactory. *Ischnura verticalis* was easiest to obtain in numbers, and since it was the most transparent of the odonatans available, it was used extensively as a metacercarial host.

3. Experiments with the definitive vertebrate host:

Metacercariae which had been watched during cyst formation were dissected from the damsel-fly naiads and fed to *Desmognathus fuscus fuscus*. The salamanders became infected with phyllodistome bladder flukes. The metacercariae excysted in the small intestine of the urodele; young worms were recovered from the posterior portion of the large intestine, the cloaca, and the urinary bladder within 24 hours after ingestion of the metacercariae.

In one experiment, a salamander was fed, at 5 P.M., one *Ischnura* naiad containing five metacercariae; at 10 P.M., the same urodele was fed another damsel-fly nymph containing two metacercariae. The next day, at 4 P.M., the *Desmognathus* was sacrificed. Three small excysted phyllodistomes were recovered from it; one worm was found in the posterior portion of the large intestine, one in the cloaca, and one in the "urethra" migrating into the urinary bladder. All these young worms still had concretions in their excretory bladders. In routine examination of the digestive system the stomach was opened. The damsel-fly which had been eaten at 10 P.M. was still being digested; one metacercaria still encysted in it was found and studied. The stylet was attached externally to a single-layered hyaline cyst wall. This fact seems to suggest the presence of an acid-pepsin labile outer cystogenous layer. The three missing young worms were not found, although the intestine, the Wolffian ducts, the mesonephroi and the coelom were examined carefully.

Worms, of increasing size, have been recovered from the urinary bladders of the salamanders 1, 2, 3, 4, 5, 15, 21, 30, 66 and 123 days after being fed metacercariae. See Figures 9–14 inclusive.

Various vertebrates have been used in experiments with these metacercariae: *Triturus viridescens viridescens*, *Rana pipiens*, *R. palustris*, *R. catesbeiana*, *R. clamitans*, *Micropterus dolomieu*, *Eupomotis gibbosus*, *Carassius auratus* and *Cyprinus sp.*, all without success.

Description of Stages in the Life-Cycle

1. Miracidium.

The ripe eggs in the metraterm of the uterus contain fully developed motile miracidia. As many as 300 free-swimming miracidia have been recovered from undiluted urine squeezed from the bladder of an infected salamander. When the sexually mature distome is placed in water, eggs are passed through the genital pore and hatch in 10 to 30 seconds. Ripe eggs of members of this subfamily usually hatch immediately in water, although Crawford (1940), for *Phyllodistomum sp.*, reported a two-day interval between egg deposition and

Plate I

Figure 1. Free-swimming miracidium.
Figure 2. Miracidium.
Figure 3. Miracidial epidermal plates.
Figure 4. Mother sporocyst (48 hours old).
Figure 5. Mother sporocyst.

FIG. 3

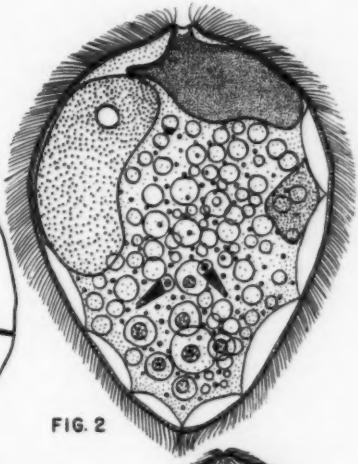

FIG. 2

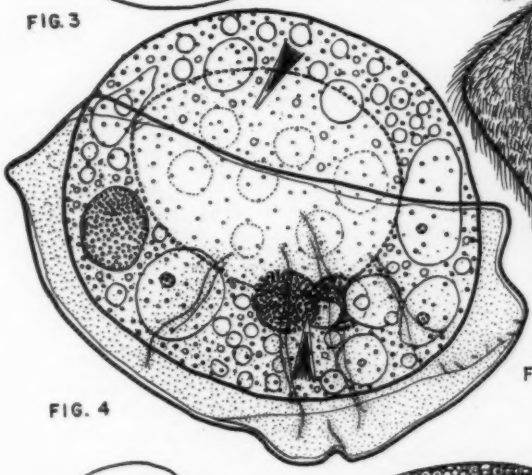

FIG. 4

FIG. 1

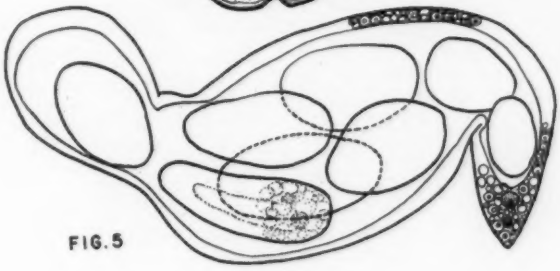

FIG. 5

PLATE 1

hatching. The free-swimming larva (Figure 1), 45 to 55 μ long by 40 to 47 μ wide, is usually mucronate-cuneate in shape, but may change to pyriform (Figure 2), often with the narrower end anterior (Figure 3). These latter shapes (Figures 2 and 3) are usually assumed when the larva is fixed or imprisoned under a cover glass.

Externally the miracidium is covered with cilia borne on 16 epidermal plates (Figure 3) arranged in three transverse rows. There are six plates in the first row, six in the second row, and four plates in the last row. Sinitsin (1905, pl. III, Figures 56 and 57) likewise indicated three transverse rows of epidermal plates in the miracidium of *P. folium*. Each cilium possesses a distinct basal granule.

Internally the miracidium is provided with an anterior sac-like "gut" 15 μ long by 24 μ wide, with a small apical opening. Situated laterally is one large gland, 34 μ long by 15 μ wide, which opens beside the "mouth." This gland is filled with refractive particles which stain deep maroon with neutral red. Rotation upon the long axis is readily observed in swimming miracidia because the asymmetrical gland appears as a rotating large, white mass. A much smaller gland, which may represent the homologue of the larger gland, is located on the opposite side of the miracidium and is often connected to the larger gland-cell by a short narrow isthmus. The body of the larva is filled with many fat-like droplets of various sizes. Similar droplets appear in all larval stages of this bladder fluke. A group of small cells with large nuclei, which may represent the germinal elements, is found in the posterior end of the miracidium. Two flame-cells are present near the posterior part of the middle-third of the body. From these flame-cells excretory ducts lead away; the ducts follow a tortuous course anteriorly and laterally for a short distance and then they turn posteriad and open separately by small lateral pores in the space anterior to the last row of epidermal plates.

2. Mother sporocysts.

The miracidia after being drawn into the mantle cavity penetrate the gills of the clam. The epidermal plates are sloughed off in one piece (Figure 4), exposing the subepidermal layer of the miracidium, which then becomes the wall of the mother sporocyst. The development of the larvae in the laboratory was slow. Four weeks after miracidial penetration the mother sporocysts (Figure 6) are small, 240 μ by 67 μ, and tubular, but well-organized germ-balls and developing daughter sporocysts are already present. From six to ten daughter sporocysts are produced by each mother sporocyst. Flame-cells in the young mother sporocyst are located in the peripheral thickened region of the larva. The

PLATE 2

FIGURE 6. Young daughter sporocyst.
FIGURE 7. Mature daughter sporocyst.
FIGURE 8. Metacercaria, encysted (12 hours old).
FIGURE 9. Young adult (one day).
FIGURE 10. Young adult (five days).
FIGURE 11. Young adult (21 days).

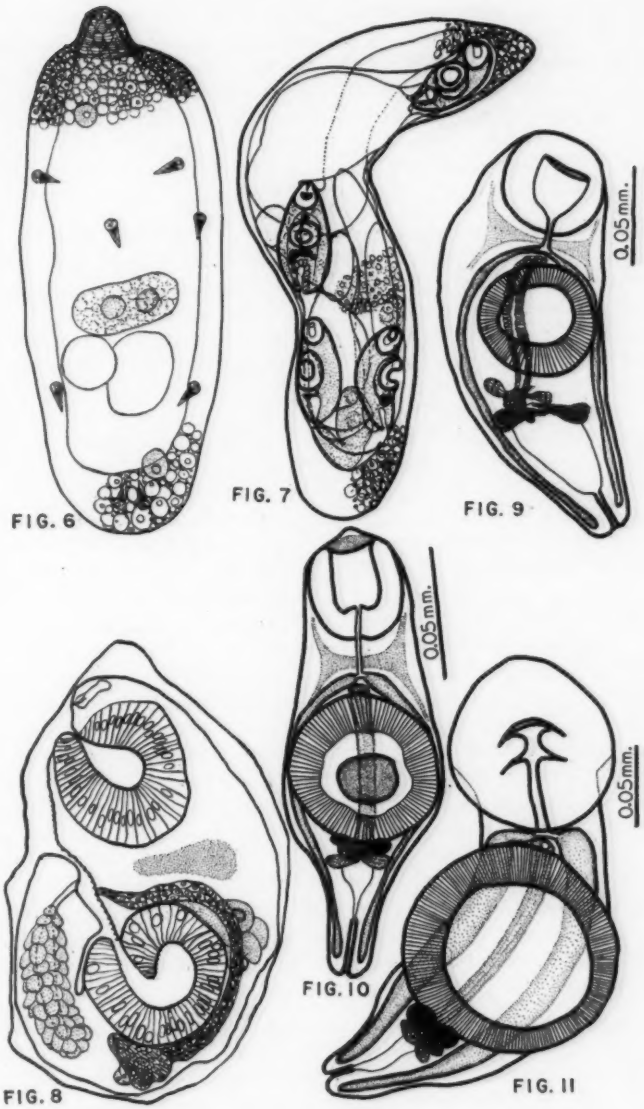

PLATE 2

sporocyst wall is relatively thick (20 μ) and is composed of clear intercellular fat-like droplets and cells with large nuclei. A six-weeks old mother sporocyst measured 490 μ by 150 μ. It had eight daughter sporocysts inside which ranged in size from about 60 μ by 50 μ for the smallest, to 102 μ by 55 μ for the largest. These young daughter sporocysts already had a wall and a central cavity containing an average of three large cell groups which may be germinal in nature (Figure 5).

3. Daughter sporocysts.

The tubular, unbranched, sluggish, mature daughter sporocyst (Figure 7) measures about 0.67–1.25 × 0.32–0.40 mm., and lies between the outermost gill plates with its constricted, evertible anterior end embedded in the gill tissue, and its posterior free end extending into the interlamellar gill space. The sporocyst wall, 27 to 50 μ in thickness, has a thin cuticula beneath which are located very thin circular and longitudinal muscle fibers. The most conspicuous elements of the wall are clear vesicular fat-droplets ranging from 5 to 17 μ in diameter. Dobrovolny (1939) reported similar structures in *Plagioporus sinitsini* sporocysts. Definite cells of two main types also occur in the wall; the most numerous are about 10 μ in diameter with nuclei 6 to 7 μ in diameter; from two to six larger cells, 15 to 20 μ in diameter, with conspicuous granular cytoplasm are also present. Many opaque granules, about one μ in diameter, occur in the sporocyst wall. Flame-cells are similar in all respects to those described by Thiry (1859), Looss (1894), Sinitsin (1905), Krull (1935) and Vickers (1940).

The interior of the sporocyst is filled with developing cercariae. The sporocyst usually contains a number of germ-balls and a maximum of seven recognizable cercariae. The large cercarial tails occupy most of the space in the sporocyst. The developmental stages of the cercariae approximate those described by Thiry (1859) for *Cercaria macrocerca*. A birth pore is located subterminally on the constricted anterior region. Cercariae can be forced by pressure through this pore; usually, however, the opening is indiscernible.

Between the sporocysts there are often numerous, opaque "yeast-like" cells of unknown origin and function, which aid in determination of infected clams *in vivo* by reflected light before cercariae are shed. The sporocysts are usually grouped in the posterior to postero-dorsal gill regions. With reflected light they appear lighter in color; with refracted light they appear as a darker mass. Unfortunately, clams which are carrying young in the marsupium present much the same appearance with both types of illumination; however, the developing young clams are located in the center of the gill region, while the parasites are located more posteriorly.

4. Cercaria.

A description of the cercaria of *Phyllodistomum solidum* has already been published by Goodchild (1939a). In this same paper a discussion of the gorgoderid cercariae was given, and a review of the known species of gorgoderid cercariae with their differences from *Cercaria Phyllodistomum solidum* (= *Cercaria conica*) was also included.

KEY TO THE GORGODERID CERCARIAE

A. Cercariae without stylets (Parasites of Unionidae and Dreissenidae)
  B. Tail shorter than body of distome..... *C. Phyllodistomum folium* Sinitsin, 1901
  BB. Tail longer than body of distome
    C. Tail with an anterior enlargement and terminal thread-like portion
      C. milocerca Miller, 1935
    CC. Tail lacks anterior enlargement and terminal thread-like portion
      D. European species..... *C. duplicata* v. Baer, 1827
      DD. American species..... *C. duplicata* of Leidy, 1858
AA. Cercariae with stylets (Parasites of Sphaeriidae)
  B. Tail inactive and very elongate
    C. Suckers of distome provided with sensory bristles; tail 4.4–7.5 mm. long
      C. Gorgodera amplicava Krull, 1935
    CC. Suckers of distome lack sensory bristles; tail 8.8–9.6 mm. long
      C. Gorgoderina attenuata Rankin, 1939
  BB. Tail active and shorter
    C. With anterior chamber containing the young distome
      D. With anterior tail enlargement
        E. Terminal filiform portion of tail shorter than anterior swelling plus anterior chamber
          C. sphaerocerca Miller, 1935
        EE. Terminal filiform portion of tail longer than anterior swelling plus anterior chamber
          F. Cercarial chamber incorporated into anterior tail enlargement
            G. Tail enlargement with lateral "wing-like" distentions
              C. raicauda Steelman, 1939
            GG. Tail enlargement without lateral "wing-like" distentions
              C. Gorgoderina vitelliloba Sinitsin, 1905
          FF. Cercarial chamber in front of and distinct from the tail enlargement
            G. Anterior tail enlargement filled with spherical cells
              H. Chamber containing distome ovoid; anterior swelling 1/10–1/13 of total tail length..... *C. macrocerca* Thiry, 1859
              HH. Chamber containing distome conical; anterior swelling 1/4–1/5 of total tail length..... *C. Phyllodistomum solidum* Goodchild, 1939
            GG. Anterior tail enlargement filled with polygonal cells
              H. Filiform portion of tail with four equidistant compact longitudinal muscle bands..... *C. coelocerca* Steelman, 1939
              HH. Filiform portion of tail without compact longitudinal muscle bands
                I. Distome with 9 pairs penetration glands; anlagen of testes in 5 parts
                  C. Gorgodera varsoviensis Sinitsin, 1905
                II. Distome with 4 pairs penetration glands; anlagen of testes in 9 parts
                  C. macrocerca Wagener, 1857
                  according to Sinitsin (1905)
      DD. Without anterior tail enlargement
        E. ..... *C. donecerca* Goodchild, 1939
        EE. Undescribed..... *C. Phyllodistomum sp.* Crawford, 1940
    CC. Without anterior chamber..... *C. macrocerca* Wagener, 1857
      according to Wagener (1857)

The presence of stylet-bearing and non-stylet-bearing cercariae in the same trematode genus is unique. In the Gorgoderidae, moreover, certain morphological and physiological relationships always accompany the stylet condition: non-stylet gorgoderid cercariae possess precociously differentiated testes, lack both penetration glands and definitely organized cystogenous gland cells around the excretory bladder, have a postacetabularly flared body, and parasitize members of the family Unionidae and Dreissenidae; non-stylet cercariae lack intermediate hosts according to Sinitsin (1901) and Reuss (1903); the adults of these cercariae are parasites of fresh-water fish. Stylet bearing gorgoderid

cercariae, on the other hand, lack precociously developed testes [except *C. Gorgodera cygnoides*, *C. Gorgodera pagenstecheri*, *C. Gorgodera varsoviensis* and *C. Gorgoderina vitelliloba* described by Sinitsin (1905)], possess penetration and cystogenous gland cells, usually lack a postacetabularly flared body, and parasitize members of the family Sphaeriidae; stylet cercariae are eaten by second intermediate vector hosts in which they encyst to form infective metacercariae; adults from stylet gorgoderid cercariae are parasites of fishes and amphibians.

All sexually mature phyllodistomes have a similar morphological pattern. Upon the basis of adult structures, it is impossible to determine the cercarial type of any adult whose life-history is unknown. If subsequent investigation proves that the adults, because of similar habitats, have converged to a common morphological form from diverse ancestry (Text Figure 1) it will be a most

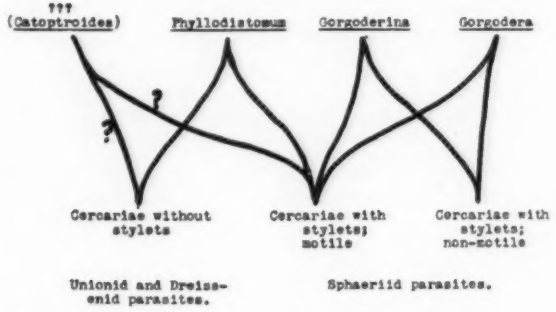

FIGURE 1. Assuming convergent evolution of adults from diverse cercarial ancestry.

striking case of convergent evolution; if the present concept of close adult relationships holds, however, which seems most likely, it must be assumed then that cercarial structures in the gorgoderids, at least, are only caenogenetic features which have been modified to fit the exigencies of their varied life-histories (Text Figure 2).

5. Metacercaria.

One hundred and forty-four damsel-fly naiads were each experimentally infected with from one to ten metacercariae. The latter are easily seen *in vivo* with intense reflected light because the opaque concretions in the excretory bladder appear pearly white. The metacercarial cysts (Figure 8) are spherical to ellipsoidal in shape, with the relatively inactive distome occupying practically all the internal space. The very delicate hyaline cyst wall (one to four μ thick) of cercarial origin is evidently later, approximately five days, augmented inside by a second cystogenous deposit of optically similar material. Vickers (1940) found, in *Cercaria macrocerca*, a group of about one dozen large cells with granular cytoplasm located antero-laterally to the penetration gland cells. He stated, "they may well be the true cystogenous cells." These cells may be the source of the secondary cystogenous material. Whether the cercaria of *Phyllodistomum solidum* possesses such cellular elements is unknown, however, because Vicker's

staining technique was not used. The host does not aid in the formation of the cyst wall, but host cellular elements do adhere irregularly to the outer cyst membrane.

The cercarial stylet which is discarded into the cavity of the metacercarial cyst during the first 24 hours, is at first free-floating in the internal liquid; later it attached to the internal surface of the cyst and is finally embedded between primary and secondary cystogenous deposits.

The growth of the metacercaria is rapid: cysts after 24 hours measured 129 $\mu \times 124\ \mu$, after 15 days 162 $\mu \times 155\ \mu$, after 30 days 170 $\mu \times 150\ \mu$, after 37 days 186 $\mu \times 150\ \mu$ and after 61 days 209 $\mu \times 202\ \mu$.

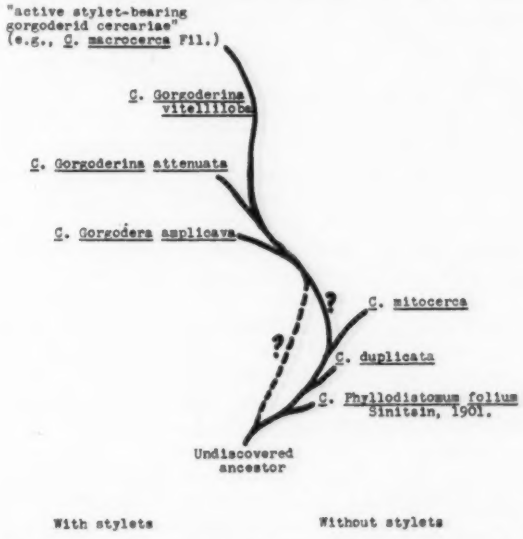

FIGURE 2. Assuming divergent evolution of cercariae from a common cercarial ancestry.

The worms can be excysted mechanically without damage to the young distome. The metacercaria retains the cercarial shape, but loses the oral and acetabular sensory bristles. Unlike *Gorgoderina attenuata*, as reported by Rankin (1939), the number and distribution of the sensory papillae remain constant from cercaria to adult. An anterior cavity in the oral sucker indicates the former position of the discarded stylet. The penetration glands with their ducts disappear and, unlike some other phyllodistomes (e.g., *P. singulare*), there are none visible in the adult. The excretory bladder and its contents are the most conspicuous structures of the aging metacercaria. Peculiar "rosette-shaped" refractive granules appear in the cavity of the bladder during the first 24 hours. These granules and the diameter of the bladder both slowly increase in size with age. At all times, however, the granules are clustered in what appears to be a cellular membrane. This membrane may be the outer wall of a "cell" which concentrates the metacercarial metabolic wastes into insoluble

concretions. The excretory granules are composed of an insoluble carbonate which gives a pink color reaction with mercuric chloride solution, and which dissolves with the evolution of a gas when treated with an acid.

Little actual progressive development of organ systems takes place during the metacercarial stage. The genital complex, the digestive system and the nervous system all remain at the cercarial level of development.

A 24 hour metacercaria, the youngest infective larva, was 0.25 mm. long by 0.11 mm. wide; the oral sucker was 62 μ in diameter and the acetabulum 64 μ in diameter; the club-shaped excretory bladder was 82 μ long by 34 μ at the widest part. A 61 day old metacercaria was 0.33 mm. long by 0.12 mm. wide; the oral sucker was 70 μ and the acetabulum 79 μ in diameter; the excretory bladder was ovoid, 124 μ long by 92 μ wide.

The length of time spent by trematode larvae in second intermediate hosts, and the degree of differentiation attained in them, often give a clue to the fundamental importance of the host. Certain cercariae (e.g., microphallids) emerge from the molluscan host in a state of relative immaturity and must remain long enough in the succeeding intermediate host to complete their development. Other cercariae (e.g., strigeids) may be apparently completely formed, but in the intermediate host almost complete dedifferentiation occurs, followed by reorganization into new and different larval forms. Still other cercariae (e.g., amphistomes and psilostomes) normally encyst in the open, or within or outside the host, but undergo few, if any, metacercarial changes.

In the gorgoderids we find what may be a partial recapitulation of such an apparent larval phylogeny. *Cercaria duplicata* of Reuss, immediately upon leaving its host, becomes a metacercaria which sinks to the bottom and lies dormant until taken in by the next host. According to Sinitsin (1901), the cercariae of *Phyllodistomum folium* encyst in the parent sporocyst which then emerges from the bivalve and is eaten by the next host. These gorgoderids have an apparent two-host cycle.

Typical three-host cycles also occur in this group of bladder worms. The cercariae of *Gorgodera amplicava*, according to Krull (1935), and the cercariae of *Gorgoderina attenuata*, according to Rankin (1939), are both passively ingested with food by snails and amphibian larvae in which they encyst as metacercariae. The cercariae of *Phyllodistomum solidum*, according to Goodchild (1939a, 1940), and the cercariae of *Phyllodistomum sp.*, according to Crawford (1939, 1940), attract and are eaten by aquatic insect larvae in which they also encyst as metacercariae. Sinitsin (1905), Lutz (1926) and Krull (1935) have also reported finding gorgoderid metacercariae in aquatic insect larvae. Progenetic gorgoderid metacercariae have been found by Wu (1938) in fresh-water shrimps. The bladder flukes reported by Joyeux and Baer (1934), in the abdominal hypaxial muscle of *Rana esculenta*, may also be precocious metacercariae. The intermediate hosts in these three-host cycles, while necessary, are hardly more than vectors which, by serving as food for the definitive host, enable easier completion of the cycle.

6. Adult.

The time necessary for this bladder fluke to reach sexual maturity in the final host is correlated with the length of time spent in the second intermediate

host; the size increase of a 61-day old metacercaria is comparable to a 15-day old young adult worm developed from a four-day old metacercaria. The temperature at which the urodele is kept may also influence the rate of development of the worm, but as yet, no controlled experimental work has been done on this aspect. These lungless plethodonids require a cool environment for satisfactory maintenance in the laboratory which may partially explain the long period required for sexual maturity of the worm (95 to 130 days).

Practically all development of the fluke takes place postacetabularly. This region increases both in length and width at a greater relative rate than the preacetabular body region.

The accompanying table of measurements (Table 1) shows clearly the increase in size of the body organs while the series of camera lucida drawings (Figures 9 to 14 inclusive) shows relative sizes and growth rates, so that a discussion of progressive development is unnecessary here.

The sexually mature distome (Figures 15, 16 and 17) can be characterized as follows: Length 1.24 to 2.67 mm., width 0.76 to 1.27 mm. The body in fixed specimens is usually not sharply divided into neck and discoidal regions. The oral sucker is large, 0.26 to 0.47 mm. long by 0.27 to 0.48 mm. wide, terminal, and cupshaped; the mouth opening is subterminal. There is no pharynx. The short esophagus, 10 to 90 μ long by 24 to 37 μ wide, bifurcates into narrow crura which immediately expand into the large digestive caeca, 105 μ to 129 μ wide, the anterior lateral edges of which often extend forward to either side of the oral sucker. Posteriorly the caeca reach approximately two-thirds of the distance from the posterior testis to the end of the body. The acetabulum, 390 to 580 μ long by 435 to 560 μ wide, is located between the anterior and middle body-thirds.

The ovary is large, 40 to 280 μ long by 160 to 280 μ wide, ovoid, usually dextral rarely sinistral, located about midway between the acetabulum and the posterior testis, and overlapping the respective gut caecum. The vitellaria consist of a pair of smooth glands lying between the acetabulum and the ovary; the right vitellarium is 78 to 199 μ long by 106 to 163 μ wide; the left vitellarium is 65 to 177 μ long by 110 to 140 μ wide. The vitellaria are connected by a large common vitelline duct, the whole yolk-gland complex appearing dumbbell-shaped. A Mehlis' gland lies at the middle of the common vitelline duct. The oviduct leaves the dorsal side of the ovary and runs antero-mediad; just before it penetrates the shell-gland (Figure 18) it enlarges to form a fertilization space filled with sperm cells; at this same point, Laurer's canal is given off dorsally; it bends anteriorly and opens to the surface dorsally to the shell-gland. The uterus loops over the vitelline duct and bends to the right, at the lateral edge of the acetabulum it turns posteriad and runs to the level of the ovary where it turns mediad between the ovary and posterior testis. The outlines of the uterus are indistinct beyond this point, and the rest of the postacetabular body is filled with developing miracidia. The uterus is visible again, running dorsad and anteriad to the acetabulum, where it expands slightly to form the muscular metraterm which opens into the genital sinus. The ovoid eggs are small, 30 to 31 μ long by 22 to 24 μ wide, and increase in size with the development of the miracidia; the shells are thin and fragile.

The smoothly contoured testes are smaller than the ovary. The anterior

testis, 124 to 168 μ long by 186 to 268 μ wide, lies usually in the ovarian field; often, however, its anterior edge may extend to or beyond a level with the anterior edge of the vitellarium. The posterior testis, 88 to 118 μ long by 270

TABLE I

Comparative measurements of Phyllodistomum solidum at various ages and from different sources
All measurements in microns

	One day	Five days	21 days	66 days	95 days	123 days	Mature adult no. 1	Mature adult no. 2	*P. solidum* ex Rankin (1937)	*P. solidum* co-type of Rankin (1937)
Length	209	170	405	513	1102	1445	1460	1240	1820–2670	2120
Width	78	75	126	260	520	685	1000	1040	760–1270	1180
Oral sucker										
Length	47	54	124	200	226	305	286	260	380–470	424
Width	48	47	130	179	243	305	286	273	380–480	453
Acetabulum										
Length	56	55	155	186	325	378	430	390	440–580	459
Width	62	59	163	205	352	390	445	435	510–560	517
Ratio oral to acet. sucker 1:	1.25	1.12	1.25	1.03	1.44	1.26	1.54	1.55	—	1.12
Esophagus										
Length	10	10	54	62	50	47	93	93	10–50	50
Width	4	4	17	18	18	22	25	24	—	37
Gut caeca										
Anterior	4	4	15	24	140	108	129	116	—	109
Posterior	4	4	15	24	109		108	105	—	
Ovary										
Length	10	9	12	39	140	160	160	112	40–280	212
Width	13	10	17	62	148	170	222	225	160–280	212
Vitellaria										
Right									Half	
Length	5	5	15	23	105	86	140	78	the	199
Width	8	7	18	40	132	99	121	163	size	106
Left									of	
Length	5	5	13	25	108	78	132	65	the	177
Width	8	8	17	39	124	93	113	140	ovary	110
Testes										
Anterior										
Length	7	7	8	30	61	110	124	127		168
Width	15	8	17	66	132	124	186	186		268
Posterior										
Length	7	7	8	47	85	128	93	88		118
Width	15	10	19	59	112	102	270	325		300
Seminal vesicle										
Length					116	80	93	108		
Width					57	62	47	50		
Eggs					28–34 ×22–24	29 ×22	31 ×22	31 ×22	30.4 ×22.4	30.7 ×23.4

to 325 μ wide, located on the ovarian side, is more attenuated transversely than the anterior testis. The vas deferens is located to the left of the metraterm; it expands near the anterior edge of the acetabulum into the conspicuous seminal

vesicle, 93 to 108 μ long by 47 to 50 μ wide. The vesicle also leads into the genital sinus which opens to the outside through the median genital pore.

Discussion

Gorgoderid bladder flukes are usually found in the urinary bladders of their hosts. In *Phyllodistomum solidum*, the young worms after excystment, emerge from the anal opening and crawl about in the cloacal cavity. Eventually they reach the urinary bladder where they reside permanently. The method of orientation of the worm to the proper cloacal opening is unknown; in *Desmognathus* it may be the action of host cloacal cilia. The manner of infestation of fish bladders by gorgoderid trematodes is still experimentally undetermined. Sinitsin (1901), in feeding experiments with metacercariae of his *P. folium*, was able to recover excysted specimens in the intestine of *Carassius vulgaris* and *Abramis brama* two hours after feeding; 24 hours after feeding, he found fewer worms in the urinary ducts. He suggested that young worms migrated from the anal to the urogenital opening; this assumption remains as a distinct probability, and the hazards of an external migration would explain the decrease in numbers of flukes found in the urinary ducts.

The mesonephroi and mesonephric ducts of larger fishes and amphibians have also been reported as additional sites for bladder flukes. Sinitsin (1901, 1905), Nybelin (1926), Lutz (1926), Odlaug (1937) and Rankin (1939) have recovered gorgoderid trematodes from these structures in fishes, toads and frogs. This fact is not surprising because the worms probably feed on the urinary epithelium. In *P. solidum*, cells of an epithelial nature have been seen in sections of gut caeca. The cercarial lytic penetration glands would undoubtedly aid in loosening the epithelial cells which may explain the retention of these glands in some gorgoderid adults. In fish, the "urinary bladder" is merely the dilation or fusion of the posterior parts of the Wolffian ducts; the epithelium is continuous and similar in both ducts and dilations.

Little can be said concerning host-specificity in the group. *Phyllodistomum folium* has been reported from *Carassius carassius*, *Barbus barbus*, *Gobio gobio*, *Scardinius erythrophthalmus*, *Leuciscus rutilus*, *L. cephalus*, *L. idus*, *Aspius rapax*, *Abramis brama* and *A. björkna*, all of which are European Cyprinidae. *P. megalorchis* has been found in *Lota lota*, *Thymallus thymallus* and *Salmo trutta*. *P. simile* has been taken from *Cottus gobio*, *C. poecilopus* and *Thymallus thymallus*. The remaining phyllodistomes are reported from only one or two host species. *P. solidum*, as previously mentioned, was recovered in only one of a group of ten vertebrate species used in feeding experiments. Rankin (1937) reviewed various expressions concerning host-specificity among helminths and stated that the North Carolina gorgoderids have an amphibian host-specificity.

The chaotic taxonomic status of the sexually mature phyllodistomes is due to synonymy and homonymy. Mature worms have few distinctive qualitative differences. Morphological structures which have been used taxonomically are: size and shape; sucker ratio; position of ovary, testes and vitellaria; size of eggs and contours of body organs. These anatomical features do not all remain constant during the maturation of members of the same species. *P. solidum*, as indicated by drawings (Figures 9 to 15 inclusive), passes from a "gorgoderina" shape to a phyllodistome one; the oral-ventral sucker ratio varies greatly in

fixed specimens due to pressure differences during fixation (Table I). The eggs likewise vary in length, from 24 to 31 μ and in width, from 14 to 24 μ, depending upon their age.

The synonymies proposed for members of this group should lead one to exercise extreme caution in future gorgoderid taxonomy. Exact taxonomic identification, without knowing the age of the worm, the physical conditions of its host, and the normal size range of the parasite, is extremely difficult. Specimens of *P. solidum* from New York State are usually smaller in all respects than those taken by Rankin in North Carolina. This fact is explained by the longer active period of the southern host, correlated with a probable one year cycle of the mature worm. From observations made in the field, it appears that salamanders are infected with metacercariae in late summer and autumn; worms develop to maturity during the remaining portions of those seasons, and during the winter and early spring. Miracidia are shed in the early spring, infecting clams which give off cercariae not before the first of July. This seasonal cycle would be extended in the milder North Carolina weather, allowing time for the worms to grow larger.

The caenogenetic organs of the cercariae offer more promise for positive specific identification. Finally, life-cycle studies, using as many different hosts as possible, may settle questionable synonymy.

Typical life-cycles were formerly believed to be family characteristics, similar in their deep-seated significance to the structures and arrangements of adult organs. In the Gorgoderinae, the life-histories so far reported, have varied greatly both in larval structures and typical sequences. The non-stylet, stumpy-tailed cercaria described by Sinitsin (1901), encysts in the sporocyst which then emerges and being remarkably buoyant because of fat-filled columnar wall cells, rises with sluggish wriggling movements to the surface, where it is eaten by fish. Dobrovolny (1939) reported the same type of life-cycle for *Plagioporus sinitsini*, a small allocread trematode from the gall-bladders of fresh-water fish. He also commented on the loss or suppression of the free-swimming cercarial stage which has been observed in other trematodes.

The non-stylet rhopalocercous gorgoderid *Cercaria duplicata* of v. Baer (1827) and Reuss (1903) emerges from its bivalve host and almost immediately the anterior portion of the large cercarial tail distends anteriorly to enclose the distome in a tail chamber. In the more typical gorgoderid cercariae, the same enclosing mechanism is effected precociously in the sporocyst. The stage represented by Reuss in Figure 4, if complete, does not represent an encysted gorgoderid metacercaria; he shows the distome body free in the tail chamber without any indication of a true enclosing cyst membrane. This fact may explain Reuss' inability to get further development of the worm in feeding experiments with any of 16 different species of fish.

Nybelin (1926) reported the encysted metacercariae of *P. megalorchis* from the fish, *Phoxinus sp.* Identification of the trematode was circumstantially based on the presence of only this one known species of sexually mature phyllodistome in the same waters. The stylet cercaria was not found.

Arthropods have also been reported as intermediate hosts of gorgoderid trematodes by Sinitsin (1905), Lutz (1926), Krull (1935), Wu (1938), Crawford (1939, 1940) and Goodchild (1939a, 1940).

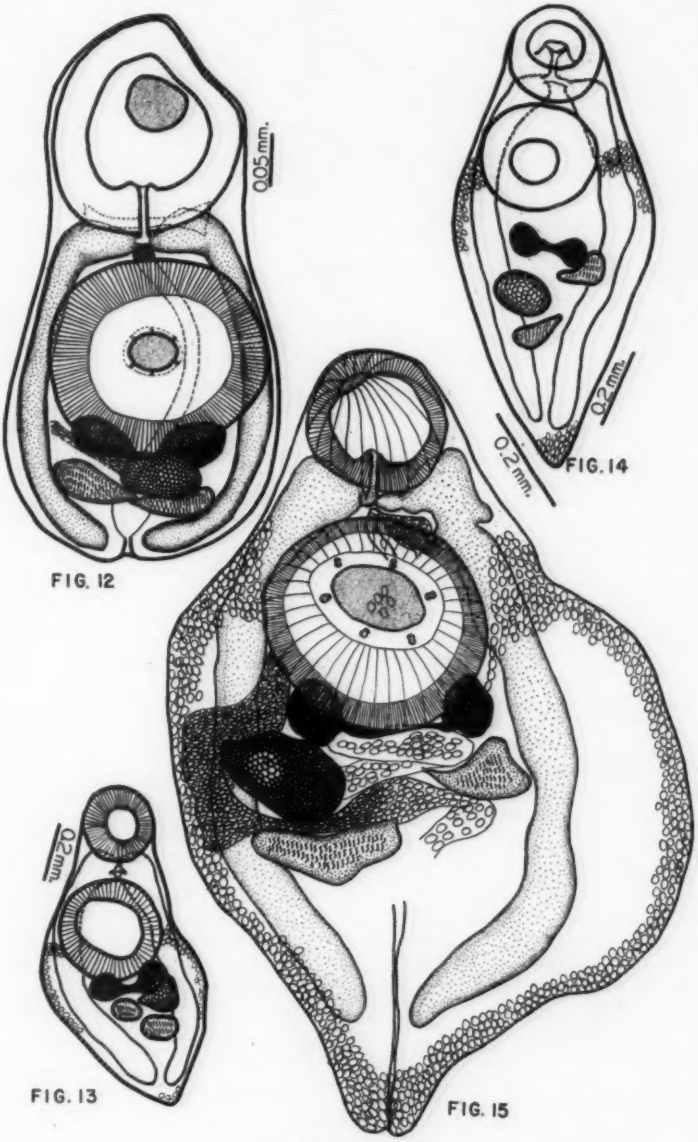

PLATE 3

FIGURE 12. Young adult (66 days).
FIGURE 13. Young adult (95 days).
FIGURE 14. Young adult (123 days).
FIGURE 15. Mature *Phyllodistomum solidum*.

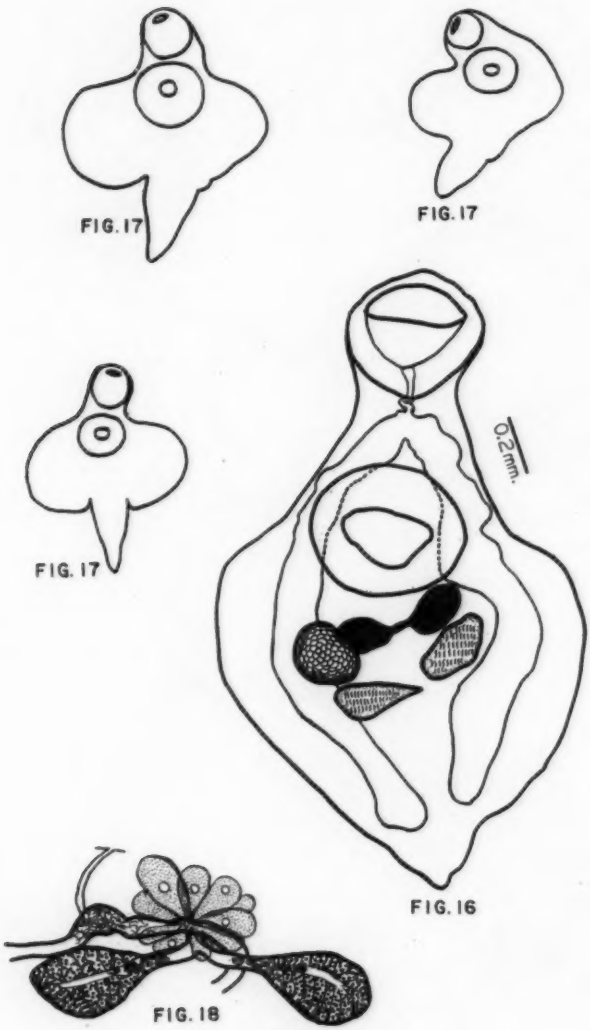

PLATE 4

FIGURE 16. Co-type, *P. solidum* of Rankin.
FIGURE 17. Shapes of living *P. solidum*.
FIGURE 18. Genital complex of adult.

Sinitsin (1905) described inclusive life-cycles for *Gorgodera pagenstecheri* and *Gorgoderina vitelliloba*, while for *Gorgodera cygnoides* and *Gorgodera varsoviensis*, he conducted incompletely controlled life-history studies. In all four of these species odonatan naiads and beetle larvae served as second intermediate hosts. Lutz (1926) reported from South America that macrocercous gorgoderid cercariae when eaten by odonatan larvae penetrated the esophagus in the wall of which they encysted. Wu (1938) reported the progenetic metacercariae of *P. lesteri* from Chinese fresh-water shrimps, *Palaemon asperulus* and *P. nipponensis;* he was unable to determine the host of the adult worm. Crawford (1939, 1940) found that odonatan larvae, caddice-fly larvae and diving-beetle larvae served as the second intermediate hosts of *P. americanum*. Goodchild (1939a, 1940, and in this paper) has reported odonatan nymphs as intermediate hosts of *P. solidum*. Krull (1933, 1935) determined that the cercariae of *Gorgodera amplicava* was ingested by the snail, *Helisoma antrosa* in which it became an infective metacercariae. Krull (1935) reported that "frog bladder fluke cercariae" were ingested by the nymphs of damsel-flies, *Lestes sp.*, and formed metacercariae in the body cavity.

Rankin (1939) determined that tadpoles of *Rana pipiens*, *R. clamitans* and the snail, *Pseudosuccinea columella* serve as the intermediate hosts of *Gorgoderina attenuata*.

Gorgoderids with two-host cycles and those with three-host cycles would appear to be distantly related. It is possible to assume, however, that there is present in this group, the fortunate retention of intermediate forms in a rather complete evolutionary series. The phylogenetic modifications and specializations of the life-cycle have not altered the fundamental morphologies of the adults or the preference for their customary parasitic sites.

Transitions Between Genera in the Gorgoderinae

1. *Gorgodera* and *Gorgoderina*.

These genera have been accepted as valid since Looss (1902) separated them on the basis of the number of testes, of which the former had nine, the latter two. Rankin (1939) showed that metacercariae of *Gorgoderina attenuata* have nine testes (six on one side, three on the other) which gradually fuse to form two in the maturing adult. In the same paper, he concluded that although these two genera of worms are definitely separable, nevertheless, "the genera *Gorgodera* and *Gorgoderina* are apparently very closely related, not only morphologically, but also with respect to their life cycles and modes of development." Krull (1935) reported that in one of the largest metacercariae of *Gorgodera amplicava* a posteriorly extended string of cells showed enlargements which were the primordia of testes. However, in young adults the, "dense testicular mass shows little evidence of being subdivided into testes, and there is little separation into right and left parts until maturity." This testicular condition again reveals the closeness of these two genera. Odlaug (1937), reporting on young kidney stages of *Gorgodera amplicava*, showed early separation into testicular masses. Although Odlaug's interpretation of the number of these units was probably erroneous, in that the ovary and vitellaria were interpreted as testes, nevertheless, the early testicular segregation that he found is significant.

2. *Gorgoderina* and *Phyllodistomum*.

For opinions regarding the identity of these two genera see: Osborn (1903), Sinitsin (1905), Cort (1912), Nybelin (1926), Ozaki (1926), Pande (1937) and Byrd, Venard and Reiber (1940). Crawford (1939, 1940) described a bladder fluke from *Bufo boreas boreas* and *Amblystoma tigrinum* which is intermediate between the two genera. This worm has a phyllodistome-like postacetabularly flared body, but also possesses prominent uterine coils between the vitelline complex and the acetabulum (*P. acceptum* and *P. marinum* have similar morphological traits), a condition regularly found in the genus *Gorgoderina*. In its early adult development this worm also passes through a typical Gorgoderina shape.

Gorgoderina schistorchis Steelman, 1938 and *Gorgoderina tenua* Rankin, 1937 possess phyllodistome morphology and they should be included in the genus *Phyllodistomum*.

Because of limited knowledge of life-histories in these genera it is unwise, at present, to suppress dogmatically one of them as a synonym of the other.

3. *Phyllodistomum* and *Catoptroides*.

For a discussion of the history of these two genera consult: Lewis (1935) and Byrd, Venard and Reiber (1940).

Nybelin (1926), Lewis (1935), Lynch (1936), Bhalerao (1937), Wu (1938) and Steelman (1938) have advocated, upon morphological bases, the suppression of *Catoptroides* as a synonym of *Phyllodistomum*. On the other hand, Loewen (1929, 1935), and Arnold (1934) upheld generic separation and described new species in the genus *Catoptroides*. Recently, Byrd, Venard and Reiber (1940), upon the basis of the excretory system, have re-established the frequently suppressed *Catoptroides*. The reasons for this action were: (1) differences in the position of the main collecting duct bifurcation (anterior to the acetabulum in the *Gorgodera-Catoptroides* group, and posterior to the acetabulum in the *Phyllodistomum-Gorgoderina* group), (2) the manner in which the collecting ducts unite with the bladder, and (3) the way in which the accessory tubules arise from the main collecting tubules.

Byrd *et al* (1940) found for *Gorgodera amplicava*, *Catoptroides lacustri* and *Phyllodistomum lohrenzi*, and Olsen (1937) found for *Gorgoderina tanneri* a constant flame-cell pattern of $2 \times 8 \times 4 = 64$ flame-cells. In their papers they did not discuss the data which has been presented concerning the bifurcation of the main collecting ducts in other species of Gorgoderinae.

The majority of gorgoderid species possess main collecting ducts which run anterior to the acetabulum before bifurcation. Sinitsin (1905) reported such a condition for the cercariae of *Gorgodera varsoviensis*, *Gorgodera cygnoides*, *Gorgodera pagenstecheri* [Wesenberg-Lund (1934) figured this last cercaria and showed bifurcation of the main collecting ducts at the level of the anterior-third of the acetabulum], and *Gorgoderina vitelliloba* [Wesenberg-Lund (1934) figured post-acetabular bifurcation in this species of cercaria, but wrote (p. 96), "two excretory canals issue from the anterior part; near the middle of the ventral sucker they seem to divide into an anterior and posterior branch."] The account given by Vickers (1940) concerning the cercaria of *Gorgoderina vitelliloba* supports the observations of Sinitsin.

The following authors have also reported an anterior bifurcation of the main collecting ducts: Reuss (1903) in *Cercaria duplicata;* Sturges (1897) in *Phyllodistomum patellare;* Krull (1935) in *Gorgodera amplicava;* Miller (1935) in *Cercaria sphaerocerca;* Steen (1938) in *Phyllodistomum brevicecum.* Wu (1938) stated that in *Phyllodistomum lesteri,* "the two tubules pass through the respective vitellaria and go around the ventral sucker." Steelman (1939) remarked for *Cercaria coelocerca,* "a pair of much-convoluted lateral collecting tubules extends anteriad from front end of bladder to near level of brain before branching." Goodchild (1939a, 1939b) found branching of the main collecting ducts anterior to the acetabulum in the cercaria of *Phyllodistomum solidum* and *Cercaria donecerca.* Vickers (1940) in a very detailed morphological investigation of *Cercaria macrocerca* Fil. (= *C. Gorgoderina vitelliloba*) described lateral excretory ducts which proceeded anterior to the acetabulum before dividing. The excretory system as described by Vickers for the cercaria is not complete, but the reported arrangement does not fit any gorgoderid pattern so far determined. Fischthal (1942) in describing *Phyllodistomum semotili, P. notropidus* and *P. nocomis* stated that the right and left primary collecting ducts are seen, "extending anteriorly to intestinal bifurcation, then looping posteriorly a short distance each receiving two secondary collecting ducts."

Postacetabular bifurcation of main collecting ducts has also been reported in a few gorgoderid species. Byrd *et al* (1940) have found this type in *Phyllodistomum lohrenzi.* Rankin (1939) reported posterior bifurcation in *Gorgoderina attenuata;* and Olsen (1937) found it in *Gorgoderina tanneri.* Walker (1937) found in *Gorgoderina vitelliloba* a median excretory vesicle extending forward to the anterior border of the posterior testis. He stated further, "here it bifurcates into two lateral canals which diverge from one another and extend forwards externally to the intestinal caeca until opposite the anterior testis, where each canal divides to form two branches, one lying dorsally to the other. The ventral branch extends forwards to the level of the acetabulum, while the dorsal extends in front of the acetabulum."

A final type of bifurcation was described by Miller (1936) in *Cercaria mitocerca.* Here, the main collecting ducts divide into an anterior and posterior branch at the level of the middle of the acetabulum.

Because there is no uniformity in the literature concerning excretory patterns of the same species (*Gorgoderina vitelliloba*), or members of the same genus, and because definite transition forms (*Cercaria mitocerca*) occur between these two different bifurcation types, it is evident that conclusions drawn now from such data are premature and questionable.

Conclusions and Summary

1. The life-history of *Phyllodistomum solidum* Rankin, 1937, a gorgoderid trematode from the urinary bladder of the urodele, *Desmognathus fuscus fuscus* (Raf.), has been completed experimentally in the laboratory.

2. *Cercaria Phyllodistomum solidum* (= *Cercaria conica* Goodchild, 1939) from *Pisidium abditum* Haldeman by vigorous cercarial activity attracts and is eaten by odonatan naiads (*Ischnura verticalis, Argia sp., Enallagma sp., Libellula sp.*) which serve as metacercarial hosts.

3. Metacercariae encyst in the thoracic haemocoele of the insect within four minutes of ingestion and are infective for the final host after 24 hours.

4. In the salamander, the metacercariae excyst in the small intestine and are recoverable in the intestine, cloaca, "urethra" and urinary bladder within 24 hours after ingestion of the infective metacercariae.

5. In the laboratory these bladder flukes require three months time to attain sexual maturity. The mature worms produce eggs which hatch immediately liberating free-swimming miracidia.

6. The miracidia are drawn by water currents into the gills of the bivalve where they transform into mother sporocysts. Mother sporocysts each produce a single generation of daughter sporocysts which, in turn, give rise to the large-tailed gorgoderid cercariae.

7. From miracidial penetration to cercarial production requires three months time in the laboratory.

8. A key to the gorgoderid cercariae is included.

9. The phylogeny and life-cycles of non-stylet and stylet gorgoderid cercariae are discussed.

10. *Gorgoderina tenua* Rankin, 1937 and *Gorgoderina schistorchis* Steelman, 1938 have been placed in the genus *Phyllodistomum*.

11. Comparisons between the genera: *Gorgodera* and *Gorgoderina*, *Gorgoderina* and *Phyllodistomum*, and *Phyllodistomum* and *Catoptroides* are discussed.

12. Observations on the gorgoderid excretory system as a basis for taxonomy are reviewed.

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THE REPRODUCTIVE CYCLE OF THE VIVIPAROUS TELEOST, NEOTOCA BILINEATA, A MEMBER OF THE FAMILY GOODEIDAE. IV. THE GERMINAL TISSUE

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INTRODUCTION

The present article is the fourth of a series written by the author on the reproductive cycle of the viviparous cyprinodont, *Neotoca bilineata*. The series has been an attempt to present a more complete study than has been available heretofore on the reproductive cycle of a viviparous teleost. Previous studies by other investigators have been confined largely either to the breeding cycle or to histological descriptions of the ovary, stressing particularly the adaptation of the ovary to the retention of the young during development. Little or no attention had been given to the actual changes in the ovarian soma during gestation until the papers on *Jenynsia* (*Fitzroyia*) *lineata* (Hylton Scott, 1928; Siccardi, 1940), *Xiphophorus helleri* (Bailey, 1933), *Neotoca* (*Skiffia*) *bilineata* (Turner, 1933), and *Cymatogaster aggregatus* (Turner, 1938b). Some of the above papers were merely introductory descriptions; others were more complete. Furthermore, with the exception of a study of the poeciliids by Turner in 1937, no reference had been made even to the cyclic variation of the germ cells during gestation. Consequently, the writer undertook this investigation with the purpose of presenting in detail an analysis of the reproductive cycle stressing particularly some of the phenomena generally omitted by previous investigators. Articles by the writer on the reproductive cycle of Neotoca have described in detail (1) the breeding cycle as observed in the laboratory (1939), (2) the marked cyclic changes in the ovarian soma during gestation (1940), and (3) the variations in the germ cell count during gestation (1941). The present article, the fourth of the series, supplements brief preliminary descriptions (Turner, 1933; Mendoza, 1938) by considering in detail (1) the general description and growth of the germ cells and their follicles, (2) fertilization, (3) the fate of the evacuated follicles, (4) the nature and fate of the atretic follicles, and (5) the origin of germ cells in the adult ovary.

MATERIALS

The present description of the germinal tissue is based on a study of over 30 ovaries in different stages of gestation. The greater part of the material was fixed in Bouin's or Zenker's fluids although fixatives such as Flemming's fluid, osmic acid, and corrosive sublimate were used for special techniques. The principal stains used were iron hematoxylin and Delafield's hematoxylin followed either by Eosin Y or Orange G. In addition, the following special stains were used: Mallory's triple connective tissue stain, Van Geisen's stain, and Foot's modification of Bieslchowsky's silver impregnation technique. The customary alcohol-xylol dehydration series was used.

Ovary

The ovary of Neotoca is a single, spindle-shaped organ inserted in the median sagittal line; it is attached dorsally to the pleuroperitoneal membrane and ventrally to the mesogaster. The ovary is continuous caudad into a single short gonoduct, a term preferred by Turner and others since, evidently, it is not homologous to the true vertebrate oviduct. Neotoca is similar to most viviparous teleosts since almost all possess the single median gonad; only in forms such as *Sebastodes rubrovinctus* (Eigenmann, 1892) is the ovary double, and only in few forms such as *Dermogenys pusillus* (Peters, 1865), *Lucifuga subterraneous* and *Stygicola dentata* (Lane, 1903), is the ovary partially fused. So far as is known to the writer, all viviparous teleosts have the single gonoduct even in forms that have the double or partially fused gonads.

Germinal Tissue

Ovigerous folds

The ovary of a teleost may be a solid or hollow organ. In the former case the eggs merely rupture the wall of the gonad, fall into the coelom and then escape to the outside through abdominal pores; in the latter type, the eggs never reach the coelom but make their way out directly from the ovary through the gonoduct. In Neotoca the gonad is hollow and the germinal tissue is said to occur internally; the same condition occurs in all other viviparous teleosts known to the writer.

The oöcytes are not scattered at random but are confined to "ovigerous" or egg-bearing folds that are placed on either side of a median sagittal septum.

Plate I

Figure 1. A cross-section of a typical non-gravid ovary (19.5×). Part of the ovarian wall at the lower left-hand corner has been removed. In the center of the figure, in a nearly vertical position is the median septum of the ovary. On either side of the septum lie the two ovigerous folds attached by a narrow, constricted stalk to the ovarian wall. The attachment is particularly clear on the left side. The arrow points to the lower edge of the ovigerous fold on the left side. Prominent in the photograph are several developing oöcytes.

Figure 2. A growing oöcyte. The photograph shows clearly the nucleolus, the vesicular nucleus, and the early follicle composed of squamous cells.

Figure 3. Two good examples of developing oöcytes and their follicles. The medium size oöcyte in the upper portion of the figure shows a nucleus that has lost its vesicular character and has become quite granular. The thickened follicle is the result of rapid mitoptic activity that does not cease until the follicle cells become so abundant they form a compact layer of columnar cells as in the egg at the bottom of the figure.

Figure 4. A figure that has a two-fold value. First, it shows an oöcyte and its follicle in a stage of development intermediate between that of the two eggs in the preceding figure. The follicle cells now are cuboidal to low columnar and soon will attain the full columnar shape typical of the full grown follicle. Secondly, the figure shows the vacuolated yolk-nucleus (arrow) in a medium size oöcyte. Figures 2 to 4 are intended to show the sequence of changes in the oöcytes and their follicles during growth.

Figure 5. A typical delle showing the funnel-like depression of the ovigerous fold epithelium that reaches to the follicle of the egg at the lower left. The intimate contact between the ovigerous fold epithelium and the follicle cells is clearly visible. Within the delle are two spermatozoa (arrows). In the upper left-hand corner is a typical mitotic figure in a growing follicle.

Figure 6. A high-power photograph (283×) of a follicle after the expulsion of the egg. The position of the former delle is visible still at the upper left-hand corner, showing the place through which the egg escaped from the follicle and the ovigerous fold. The follicle cells still retain their peripheral position although degenerative changes already have started.

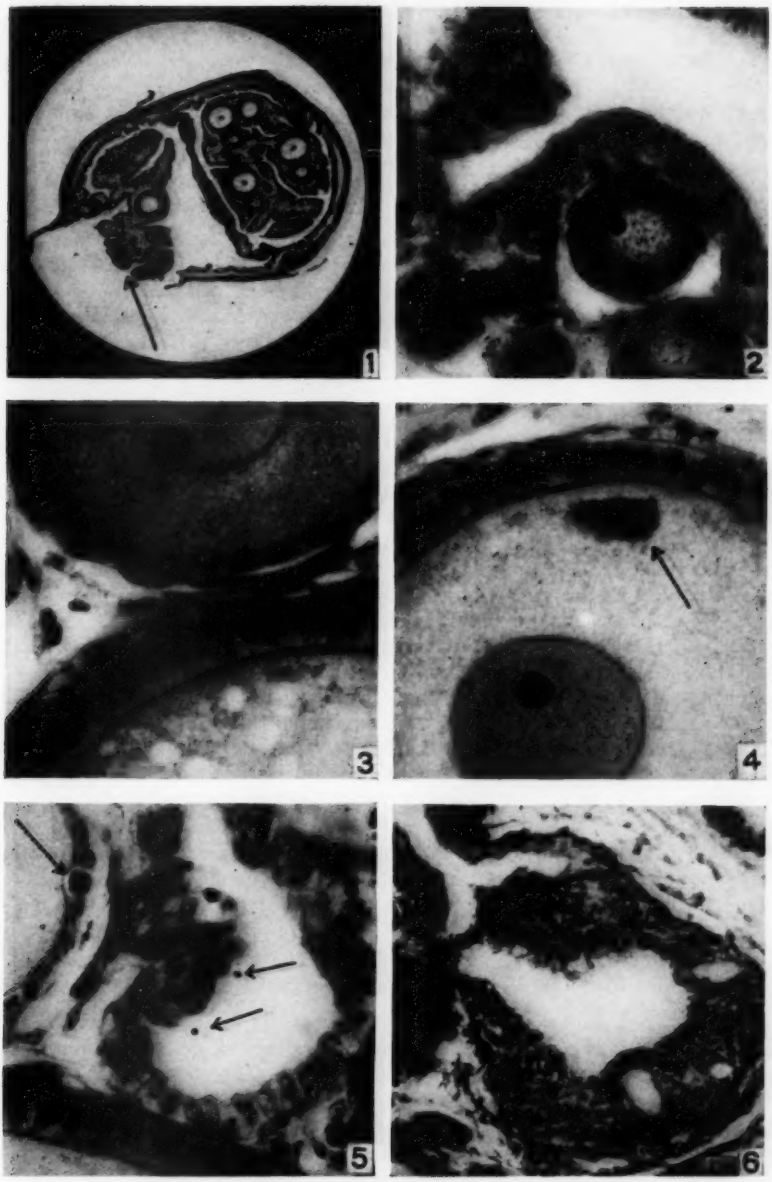

PLATE I[1]

[1] All figures are contact prints of unretouched photomicrographs. Figure 1 was taken with a Reflex-Korelle camera; all others were taken with a Voigtlander cut-film camera. With exception of Figures 1 and 6, all other photographs were taken with a fluorite, oil-immersion objective and a Hyperplane ocular; the magnification of the oil-immersion photographs is approximately 650×.

Each fold is attached by a narrow strip along the dorso-lateral wall of the gonad; the two folds virtually fill the ovarian lumen (Figure 1). Internal ovigerous folds by no means are characteristic of all viviparous teleosts since *Xiphophorus helleri*, *Anableps anableps*, and *Zoarces viviparus* are devoid of special folds; the ova merely develop in the walls of the ovary. On the other hand, forms such as *Cymatogaster aggregatus*, *Dermogenys pusillus*, *Jenynsia lineata*, *Sebastes marinus*, etc., resemble Neotoca and other members of the Goodeidae by the presence of one type or another of ovigerous folds or septa. In fact, these folds are so diagnostic in the Goodeidae that Hubbs and Turner (1939) used these same folds, in part, as a basis for their recent taxonomic revision of the family.

Histologically, the folds consist of a loose, collagenous connective tissue that in part shows a marked affinity for argyrophilic stains. A vascular network ramifies throughout the folds and forms a very rich capillary plexus under the epithelium of the folds. It has been shown (Mendoza, 1940) that during gestation, this internal epithelium covering the folds changes from a flattened, indifferent condition to a tall, highly secretory epithelium. Parallel with these changes, the folds likewise become distended with fluid and are invaded by numerous free cellular elements that contribute to the general tumescence of the ovary during gestation. Within the ovigerous folds of Neotoca, the germ cells are evenly distributed throughout the length of the gonad except at the extreme anterior and posterior ends. The growing germ cells are not scattered at random but are normally grouped into clusters of cells that range from minute oöcytes 7 to 10 μ in diameter to fully grown eggs approximately 200 μ in diameter. In addition, these clusters of cells normally occur at the surface of the folds, retaining a very intimate connection with the epithelium (Figures 2, 10, 12), a relationship that was stressed also for Anableps by Turner (1938a). At this point of contact between the fully grown ovum and the epithelium, there appears a deep, funnel-like depression of the ovigerous fold epithelium. The cells at the base of the pocket are usually flattened strongly against the follicular cells surrounding the ovum (Figures 5, 9). Normally this depression does not occur at the outer surface of the folds but at the base of one of the many fissures that crease the surface of the folds. This funnel-like depression has been described in other viviparous teleosts and has been identified as a "tubular indentation," a "follicular pore," or a "delle." The two-fold function of this structure will be discussed elsewhere in this paper.

Germ cells and follicles

Germ cells are recognizable when they are but 7 μ in diameter and are undergoing early stages of maturation. Such early cells are grouped into small nests and usually are attached closely to the ovigerous fold epithelium. In these early cells the nucleus is vesicular and occupies fully two-thirds or more of the diameter of the growing oöcyte. The cytoplasm shows an affinity for acidophylic stains and is heavily but evenly granular (Figure 2). The early follicle consists of a few delicate, squamous cells flattened against the surface of the oöcyte (Figure 2); because of the manner of formation of the germ cell nests and the structure of the follicle cells, it is likely that the latter are nothing more than modified fibrocytes of the subepithelial connective tissue.

In the growing oöcyte, furthermore, many changes occur. The nucleus is reduced to one-third of the diameter of the cell and the vesicular character is lost; it becomes granular and even oxyphylic in nature (Figures 2 to 4). During growth, the chromatin loses all affinity for stains although it later reappears in the form of lampbrush chromosomes. Whereas in the early cells the nucleoli may be numerous, less than a micron in diameter and strongly basic in reaction, later they are but few in number, large, vacuolated, and even oxyphylic in their reaction to stains. In general, however, their number, size, and appearance are highly variable in the different cells and at different stages of development. In the growing cells the cytoplasm also undergoes marked changes. The most conspicuous characteristic of the oöcytes is the complete absence of large masses of yolk and the presence of numerous small droplets of oil scattered throughout the granular cytoplasm. The yolk, such as it is, consists largely of a granular, flocculent mass evenly distributed throughout the cytoplasm and very difficult to distinguish except with the use of differential stains and particularly in stages immediately following fertilization. There is not the least similarity to forms such as Zoarces in which the yolk appears as large spheres, nor to Jenynsia and Xiphophorus in which the yolk appears as a large solid mass. Furthermore, another goodeid, *Lermichthys multiradiatus*, shows a heavy yolk mass in the developing embryos, a mass that, though small in size, is so heavy and compact, it cannot in any way be compared to the "yolk" of Neotoca. It is regrettable that lack of material prevented further testing or differentiating of fatty and proteinaceous yolk in Neotoca although the use of Flemming's fixative on the ova of another goodeid, *Girardinichthys innominatus*, showed beyond doubt that there is a large number of droplets or spheres of fatty yolk concentrated around the nucleus of the ovum. Another conspicuous feature of the fully-developed ovum is a large, vacuolated structure that is very similar to or forms a yolk-nucleus and pallial layer complex (Figure 4). The origin, development, and fate of this yolk-nucleus complex is so interesting in these viviparous forms that it will be discussed at length in a separate paper. It is interesting that, among viviparous teleosts, Cymatogaster and Jenynsia have yolk nuclei equally as prominent as that of Neotoca. The egg membranes of the enlarged oöcytes are not numerous; there is primarily one heavy vitelline membrane densely perforated in part, if not in its entirety, by minute pores. Thus it is similar, though not identical, to the thick, perforated, zona radiata described for Zoarces by Stuhlmann and for Cymatogaster by Hubbard (1894). Internal to this heavy egg membrane is a more delicate plasma membrane discernible only after fertilization when the heavy outer membrane is separated somewhat from the egg. During growth of the oöcyte, the mitotic activity of the follicle cells not only keeps pace with but actually surpasses the growth of the egg since the follicle changes from a delicate layer of flattened cells to a single, densely-packed row of columnar cells (Figures 2 to 5), a follicle that more closely resembles that of Xiphophorus and the early follicle of Anableps. In comparison, follicles of a compound nature are found in forms such as Jenynsia, Stygicola, Lucifuga, and Cymatogaster. The follicle in Neotoca is in turn invested by a thin layer of condensed connective tissue fibers and fibrocytes comparable to the "theca" described by Bailey for Xiphophorus. The connective tissue fibers of this "theca" are intimately associated with those of the subepithelial network of the

ovigerous folds and, like reticular fibers, similarly show a marked affinity for argyrophylic stains. Interspersed throughout the interstices of this network there occurs a vascular network more or less prominent in the different follicles and presumably of great importance in the physiological activity of the follicle of the developing oöcyte.

Fate of the Germ Cells

After the ova have attained full growth they suffer one of two fates, either they are fertilized and commence development or they undergo atresia. Both possibilities are considered.

Fertilization

Among viviparous teleosts, fertilization may occur while the egg still is retained within the follicle or after its extrusion into the ovarian lumen. In forms such as Anableps and Xiphophorus, fertilization occurs within the follicle and the embryo is not released until a short time preceding birth whereas in Jenynsia, fertilization is similar but the embryo is discharged early in development. On the other hand, in Cymatogaster, *Sebastes marinus* (Williamson, 1910), and Neotoca, fertilization and the expulsion of the egg must occur in such rapid succession that it is difficult to separate the two phenomena. Finally, in Zoarces, eggs are discharged first into the ovarian lumen and fertilized later. Accompanying these varying conditions of fertilization, structural and functional problems are presented by the different types, problems that are largely beyond the scope of this paper.

Preceding fertilization in Neotoca, the different stages of meiosis can be identified readily. Following synizesis which appears in oöcytes 7 to 10 μ in diameter, the chromatin temporarily loses all affinity for stains and later reappears in the form of lampbrush chromosomes. Immediately preceding actual fertilization the chromatin condenses markedly, resembling stages of diakinesis, while the entire germinal vesicle migrates simultaneously toward the periphery of the egg. This migration occurs normally in the direction of the delle, in preparation for the ensuing meiotic divisions and fertilization. Evidently, the meiotic divisions, fertilization, and the expulsion of the egg into the ovarian cavity must occur simultaneously or in very rapid succession, for none of the three phenomena actually have been seen although all stages immediately preceding and following their occurrence have been identified. Unfertilized eggs always have been identified within the follicles; on the other hand, fertilized eggs normally have not been seen within the follicles but always free in the ovarian lumen. The criterion used to distinguish fertilized eggs has been the very radical displacement of cytoplasmic components within the egg and the ensuing segmentation. In these eggs, the cytoplasm is concentrated as a thin peripheral layer, presumably along the animal pole; small oil droplets occur evenly distributed throughout its extent. Underneath the thin blastodisc, the central portion of the fertilized egg is filled by a flocculent, albumen-like substance which apparently replaces the heavy yolk of other viviparous eggs. Occasional spheres of actual yolk can be found, but they are minute and extremely scarce. Although the meiotic divisions

have not been seen, the polar bodies have been identified on the edge of the heavy vitelline membrane. A unique phenomenon is that in the vicinity of the polar bodies the vitelline membrane not only shows a strong affinity for basic stains but also thickens conspicuously at time of fertilization to resemble the typical zona radiata of teleost eggs. Since this thickening of the membrane occurs only in evacuated eggs and only near the polar bodies, it is assumed that it arises in response to the inciting action of the spermatozoa. Due largely to the swelling of the vitelline membrane of these eggs, it can be distinguished from the egg with ease.

It is at the time of fertilization that the delle formed by the ovigerous fold epithelium plays an important role. Through the delle, the spermatozoa actually can approach the egg very closely (Figures 5, 9). If the sperm do, in fact, enter the egg while the latter still is within the follicle, the sperm then must penetrate only through the single row of flattened cells at the base of the delle and the row of columnar follicle cells. However, the writer never has identified an actual pore within the follicular epithelium to permit the entry of the spermatozoa as described for Xiphophorus by Bailey. Evidence is available from other viviparous teleosts to show that fertilization within a follicle can and does occur. In Xiphophorus, Anableps, Cymatogaster and Jenynsia, eggs are fertilized while still enclosed within the follicle and later are discharged into the ovarian lumen at different stages of development. The second and final function of the delle is that it offers an attenuated and weakened place in the ovigerous fold epithelium through which the egg escapes into the ovarian cavity. A similar role for the delle has been reported for other viviparous teleosts in which it appears.

Upon evacuation of the egg, the follicular cells cease all mitotic activity and suffer one of two or three fates. Sometimes the force of the expulsion of the egg is so great that the follicle cells may be everted part way through the delle. Normally, however, the follicle cells either merely collapse to form a cellular mass of smaller diameter or the follicle cells may retain their peripheral position around the reduced space previously occupied by the egg (Figure 6). Despite these differences in evacuated follicles, the cells eventually lose their regularity and become a disorganized mass of cells. The evacuated follicle does not assume the "windswept appearance" of the Xiphophorus follicle (Bailey) nor does it become hypertrophied as in *Sebastes marinus* and *Anableps anableps*. It is impossible to compare the spent follicles of Neotoca with the mammalian corpora lutea since in Neotoca there is no evidence of a pronounced physiological activity; actually, the follicle cells are small and shrunken. Pycnotic figures of degeneration make their appearance soon after the extrusion of the egg; vacuolization and fatty degeneration have been found among these follicles (Figure 6). By the time embryos are 1.5 mm. in length it is difficult to distinguish between spent follicles and ordinary atretic follicles. It is to be expected, perhaps, that in Neotoca the follicle should be passive and soon degenerate for, since the embryo develops completely within the ovarian lumen, the follicle is relieved of all responsibility of serving the developing embryo in a nutritive or respiratory capacity. Thus the Neotoca follicle is not taxed with a physiological burden similar to that of Anableps nor poeciliids like Xiphophorus in which the embryo develops completely within the follicular sac.

Atresia of the eggs and follicles

Egg degeneration is found in all ovaries. It is evident that eggs degenerate rapidly if not fertilized soon after they have attained full growth. As many as 25 eggs in various stages of atresia have been found in a single non-gravid ovary. In typical cases of degeneration which occur during all stages of gestation, both the follicle and the egg are involved. Normally, the egg and especially the cytoplasm is the first to disintegrate; the alveolar structure is lost and frequently the cytoplasmic residue coagulates upon fixation. Coexistent with these changes, the follicle cells lose their regularity, mitosis ceases, the vitelline membrane is thrown into folds, and the follicle cells are displaced toward the atretic egg. Normally, however, the follicle cells retain their peripheral position for some time while the cytoplasm of the egg breaks up. It is likely that the debris of the egg is removed in part by absorption and in part by actual phagocytosis. With the removal of most of the cytoplasmic residue, the follicle collapses completely and forms a nearly solid group of cells surrounding a mass of debris (Figures 7, 8). During this process, slight lymphocyte infiltration usually occurs and may be instrumental in aiding the process of disintegration. In the follicle cells, the nuclei are the first to undergo pycnotic degeneration; the breakdown occurs gradually, cell by cell. The degeneration picture most definitely is not one of complete breakdown of all or most cells at once. In a few isolated cases an abnormally heavy lymphocyte infiltration may occur and in others the follicle may precede the egg in degeneration. These, however, are infrequent in their occurrence. Eventually, regardless of the method of atresia, the end result is the removal of the degenerating mass of cells from the stroma of the ovary.

Origin of the Germ Cells

Although no study has been made of the origin of germ cells in the embryonic gonad, the writer feels that there is available interesting evidence on the origin of the cells in the adult gonad. The observation has been made repeatedly that

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Plate II

Figure 7. An atretic follicle showing the disorganized state of the follicle cells, the cellular debris, some fatty degeneration, and several large vacuoles that have appeared between the cells.

Figure 8. A degenerating follicle that has been reduced to a small cellular mass. In the center there occurs some cellular debris and one atretic cell (arrow).

Figure 9. This and the remaining figures on the plate have the single purpose of showing the frequent occurrence of growing oöcytes in the ovigerous fold epithelium and the possible origin of some germ cell nests from the epithelium. The arrows indicate clearly an oöcyte in the epithelium that lines the delle and three spermatozoa within the delle proper. This and Figure 5 show the relation of the ovigerous fold epithelium of the delle to the egg and its follicle.

Figure 10. The arrows indicate first, a developing oöcyte in the ovigerous fold epithelium and second, a new germ cell nest apparently derived from the epithelium.

Figure 11. A single, distinct oöcyte in the epithelium of the ovigerous folds. The subepithelial connective tissue, showing black in the photograph, indicates clearly that the germ cell is in the epithelium and not merely lying against it.

Figure 12. The opening that shows in the center of the photograph is a cross-section of a delle. The arrows indicate two oöcytes within the epithelium of the delle and other germ cells that apparently have broken through the subepithelial connective tissue fibers to form a small clump or nest of growing oöcytes. The photograph shows clearly how the basement membrane has been ruptured completely at this point, indicating a complete continuity between the growing oöcytes and the ovigerous fold epithelium.

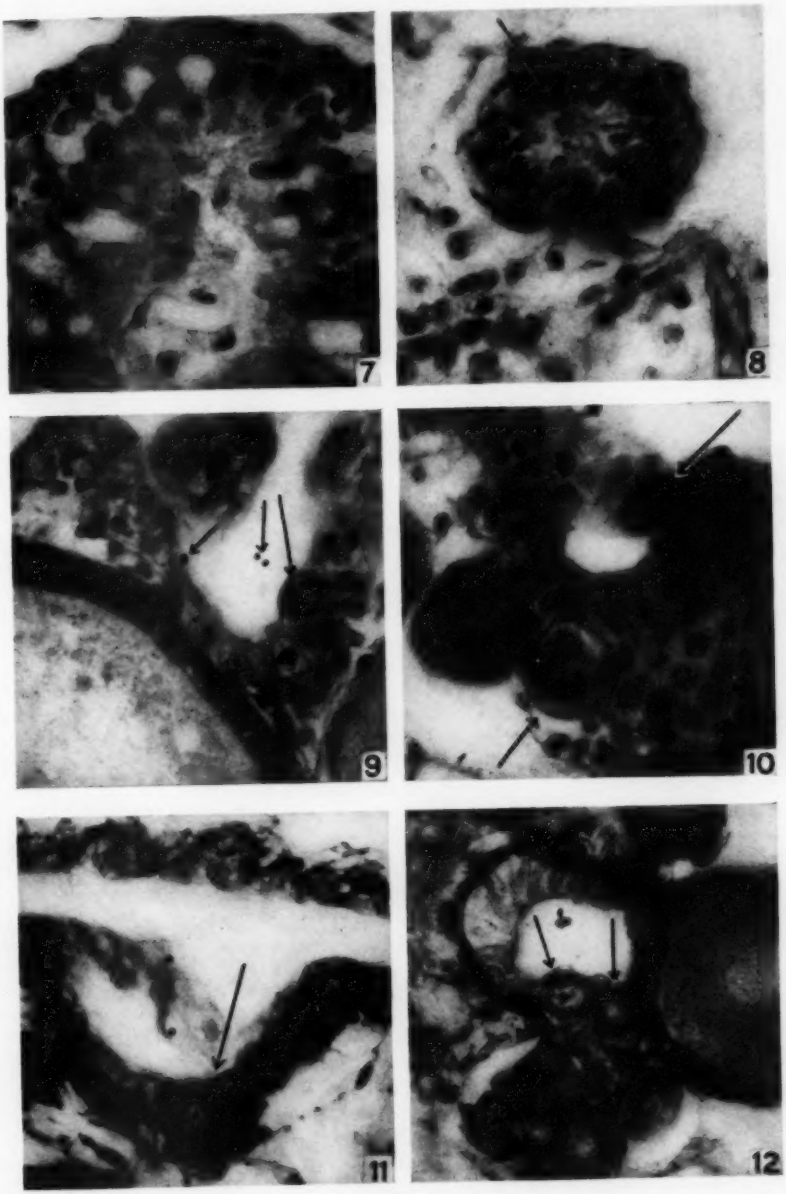

PLATE II [2]

[2] See footnote 1.

cells normally occur in clusters at the surface of the ovigerous folds (Figures 2, 10, 12). This is true particularly at the interlobular fissures that occur in the ovigerous folds. The attachment of the full grown oöcytes to the ovarian delle has been described in another part of this paper. Histologically, it is evident that the subepithelial connective tissue fibers are in direct continuity with those surrounding the large individual cells and clusters of small cells (Figure 10). Furthermore, there are frequent examples of prominent invaginations of the ovigerous fold epithelium to form small nests or clusters of epithelial cells. These invaginated nests to all purposes appear like clusters of typical gonial cells (Figures 10, 12). In addition, typical growing oöcytes frequently appear in these invaginated cell nests (Figure 12). The striking feature of many of the nests is that the cells still are in direct continuity with the epithelial cells on the surface of the ovigerous fold; in other words, the invaginated cluster of cells has not been pinched off as yet from the superficial epithelium. With differential stains, it is possible to determine that the invagination of the cells does not always break through the underlying, subepithelial connective tissue fibers but rather that the heavy fibers and the accompanying fibrocytes are carried along with the invagination to form a thin connective tissue sheath around the nest of cells. Thus, in these cases, not only the cells within the nests but also the connective tissue fibers around the nests are continuous with the corresponding elements at the surface of the ovigerous folds. These nests then are pinched off from the surface. Lastly, the observation has been made frequently that oöcytes may occur within the epithelium proper of the ovigerous folds. They are much larger than the adjacent epithelial cells and have the customary large vesicular nucleus (Figures 9 to 12). A secondary migration of these oöcytes into the epithelium appears unlikely. Rather, it appears that these are examples of epithelial cells differentiating *in situ* to form germ cells. In these cases, differentiation occurs without the usual invagination. Therefore, in view of (1) the intimate histological connection between the oöcytes and the ovigerous fold epithelium, (2) the actual invagination of the epithelial cells, and (3) the occurrence of typical oöcytes in the epithelium proper, the writer is firmly convinced that, in the adult gonad at least, some of the germ cells arise from the ovarian epithelium on the ovigerous folds. It is interesting that Turner made similar observations in the ovary of *Anableps anableps*. He not only stressed the subepithelial position of the germ cell nests and oöcytes but also noted occasionally single oöcytes in the epithelium itself. However, he arrived at no conclusions regarding the origin of the germ cells.

Summary

1. Two ovigerous folds, one on either side of the median sagittal septum of the ovary are described as bearing the germ cells.

2. Oöcytes normally occur in clusters at the surface of the ovigerous folds.

3. Follicular pores or delles not only facilitate access of the spermatozoa to the ovum but also provide a place for the escape of the fertilized egg.

4. The growing oöcyte is characterized largely by the absence of large masses of yolk and the presence of numerous oil droplets. During growth the nucleus changes from a typical germinal vesicle to a granular, eosinophylic body in which the chromatin exhibits only a weak affinity for stains.

5. The follicle of Neotoca changes during growth from a tenuous layer of scattered squamous cells to a thick, simple layer of columnar cells.

6. Fertilization, the completion of the meiotic divisions, and the escape of the egg are described as occurring simultaneously or in extremely rapid succession.

7. Normally evacuated follicles cannot be compared in any way to the mammalian corpus luteum.

8. In the atresia of the follicles the following phenomena are believed to occur: some fatty degeneration, some liquefaction or vacuolization of cells, some lymphocyte infiltration, some phagocytosis, and lastly, some absorption by the surrounding cells.

9. Some, if not most, of the germ cells of the adult gonad of the female are believed to arise from the epithelium of the ovigerous folds.

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THE REACTION OF CERTAIN CRUSTACEA TO DIRECT AND TO DIFFUSE LIGHT

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Many plankton organisms which move downward, away from daylight in the sea, move toward a source of light in the laboratory. It was suggested in a previous paper (Schallek, 1942) that this behavior is caused by the animals' being exposed to diffuse light in nature, but to direct light in the laboratory. It was reported that when a tall glass cylinder containing the copepod *Acartia tonsa* was illuminated from above, the animals swam up toward the light. This phototropic reaction seemed to depend on the fact that the light was shining directly down the axis of the cylinder. But when the lamp was moved so as to illuminate the container obliquely, the refraction of the light at the surface of the water and its reflection from the curved inner wall of the cylinder seemed to form a diffuse illumination. Under these circumstances the animals sank downward, simulating their behavior in nature. The present paper provides a further development of this hypothesis based on measurement of the light distribution inside the cylinder.

Apparatus

A glass cylinder 18 inches high and six inches in diameter was used for the experiments. The cylinder was kept at 15° C. in a constant temperature bath 15 inches high and 15 × 18 inches in area. The bath had black walls to absorb reflected light, and was kept in a photographic darkroom. Illumination was from a 75-watt lamp with a parabolic, aluminum-coated reflector. The lamp was placed in five different positions, each 12 inches from the top of the cylinder, but separated by 22.5°. Position 1 was on a level with the top of the cylinder, while position 5 was directly overhead.

Light measurements were made with a Westinghouse "Photox" cell connected to a micro-ammeter, and calibrated in foot candles with a Macbeth illuminometer. The cell was placed in a waterproof case, and covered with a cylindrical hood, limiting the light received to an angular opening of 22.5°. The cell was held six inches below the surface of the cylinder by clamps and rods attached to a ringstand. As this apparatus would not fit inside the six-inch cylinder, an 11-inch cylinder was used for the light measurements. This change is not believed to be a significant source of error.

Twenty *Acartia tonsa* were placed in the cylinder at a time, while the lamp was shifted from position 1 (oblique) to position 5 (overhead). Alternate runs were made in the opposite direction. Two hours' exposure to each position was allowed before counting to permit the animals to reach equilibrium. Results are presented as the percentage of animals in the top third of the cylinder.

[1] Contribution No. 323.

Observations

A. tonsa moves down when the illumination is oblique, and moves up when the lamp is overhead (Table I). Measurements of the light distribution inside the cylinder are given in Figure 1.

Table I

Distribution of animals in cylinder
Average of three runs with 20 animals each

Light position	1	2	3	4	5
Elevation above horizontal	0	22.5	45	67.5	90°
	Per cent animals in top third of cylinder				
Acartia tonsa	13	29	47	65	68
Centropages typicus	69	70	72	72	79

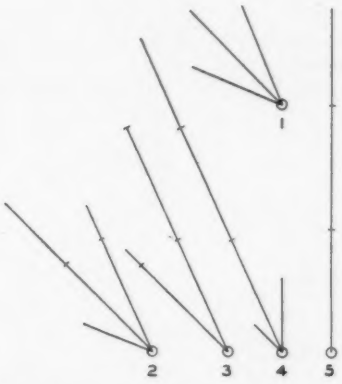

Figure 1. Light distribution inside the cylinder for each position of lamp. The small circle indicates the location of the photometer, six inches below the surface of the water. The number of each lamp position is given below the circle. The length of each line represents the light intensity recorded when the photometer was pointed in that direction. Logarithmic scale: the first crossbar = 100 foot-candles, the second = 1000 F. C. The hood limiting the incident light to 22.5° made the cell insensitive to less than 20 F. C.

These measurements reveal several factors that may influence the behavior of *A. tonsa:*

1. There is a 50-fold increase in the *intensity* of the maximum beam as the lamp is raised over the cylinder. It may be that the animals swim up in a bright light and sink down in a dim one. To test this factor, a slide-wire resistance was connected in series with the lamp, permitting the light intensity to be varied while the lamp was held in the overhead position. A 120-fold increase in the intensity of the light had no effect on the distribution of *A. tonsa* (Table IIA).

2. The *direction* of the maximum beam is vertical in position 5, but is more horizontal in the other positions. Perhaps the animals swim toward a vertical light but sink downward in a horizontal one. This possibility was tested by

TABLE II

Effect of changing light intensity

A. Cylinder vertical, light overhead.

Average of three runs with 20 *A. tonsa* each

Light intensity	10	200	1200 F. C.
Per cent animals in top third of cylinder	56	52	54

B. Horizontal trough, illuminated from end.

Light intensity		10	20	200	1300 F. C.
Time to move 10 inches					
Animal	1	130	150	125	130 seconds
	2	15	25	10	15

placing the cylinder on its side. The light from position 5 (shining directly down the axis of the cylinder) formerly was vertical, but now came in a horizontal direction. When the lamp was in position 1 (oblique) the animals sank downward (Figure 2A); when it was moved to position 5 (now horizontal) the animals swam to the end of the cylinder nearest the light (Figure 2B). Hence the animals swim toward the light when it is parallel to the axis of the cylinder, and sink downward when it is oblique, regardless of whether the light comes from a horizontal or a vertical direction.

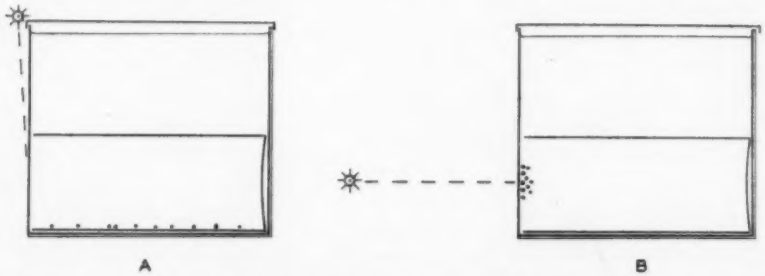

FIGURE 2. Arrangement of apparatus to test effect of light direction. A, lamp in position 1; B, lamp in position 5. Position of animals shown by black dots. The cylinder is on its side in a glass tank painted black (double line) on all sides except one through which the light enters. A piece of tarpaper covers the top.

A further test was made with a horizontal tropism trough, which could be illuminated at either end by a beam of light shining directly down the trough. *A. tonsa* always moved toward the light under these conditions; by turning on first the light at one end and then that at the other, it was possible to keep an individual moving back and forth from end to end indefinitely. Here again the reaction is independent of light intensity (Table IIB).

3. The *distribution* of the light changes as the lamp is moved. In position 5 the illumination is highly directional, with all the light coming from a single direction. In position 1 the illumination is more diffuse, the light coming almost equally from three directions. This angular distribution can be conveniently measured by the ratio (sum of intensities in other directions) : (highest intensity).

The 22.5° hood permitted 16 readings to be taken by rotating the photometer through 360°. For perfectly direct illumination, with all the light coming from one direction, this diffusion ratio will be 0 : 1, or 0. For perfectly diffuse illumination, with the light coming equally from each of the 16 directions in which measurements were made, it will be 15 : 1, or 15. The diffusion ratio for each position of the lamp is given in Table III. The curve formed by plotting these values against the percentage of animals in the top third of the cylinder is shown in Figure 3.

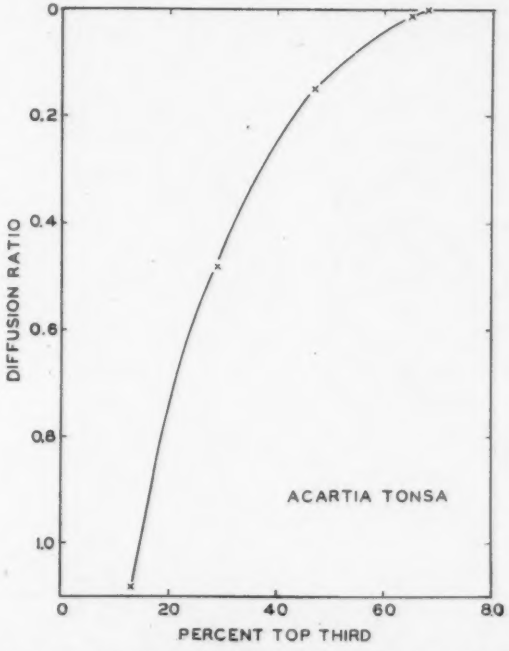

FIGURE 3. Influence of distribution of light on behavior of *A. tonsa*. Ordinate, diffusion ratio of light. Abscissa, per cent of animals in top third of cylinder.

The possibility remains that this relation is an artifact caused by the particular apparatus or procedure used. This is not so, since another copepod, *Centropages typicus*, is only slightly affected by the shift in lighting (Table I). It must therefore be concluded that the behavior of *A. tonsa* depends upon the angular distribution of the light:

1. *A. tonsa* moves toward a source of highly directional light, regardless of the intensity of the light or the direction from which it comes. This is a typical positive phototropism.

2. *A. tonsa* sinks downward in less directional (diffuse) light. This is a positive geotropism and not a negative phototropism, since the animal does not move along the beam of maximum light, but sinks passively downward.

Which of these types of illumination will animals encounter in nature? Measurements were made of light distribution in air, while the distribution of light in the sea was calculated from the data of Whitney (1941) (Figure 4, Table III).

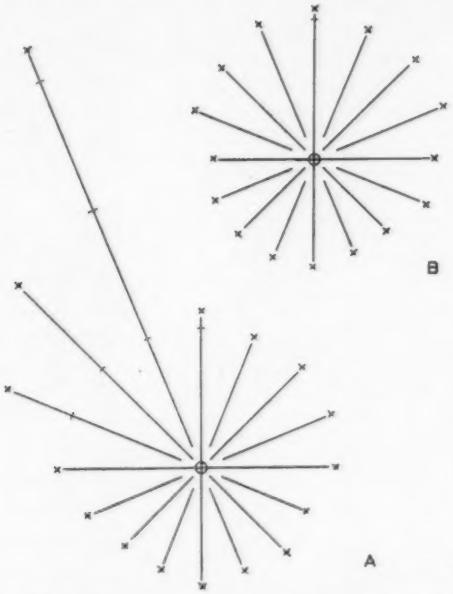

FIGURE 4. Light distribution three feet above roof of Marine Biological Laboratory. August 2, 1942, 10 A.M. Arrangement as in Figure 1.
A. Clear sky, plane of sun.
B. Clear sky, plane perpendicular to sun.

It is evident that light in the sea becomes even more diffuse than any conditions reached in the cylinder. *A. tonsa* sinks downward in diffuse light in the laboratory, and this reaction apparently accounts for the fact that it leaves the surface of the sea in the daytime (Esterly, 1928). *Centropages typicus*, which moved down only slightly in the diffuse light in the cylinder, shows only a slight downward movement in the sea (Clarke, 1934).

DISCUSSION

There are several reports in the literature of animals which react phototropically in a horizontal tube illuminated from the end, but which sink downward in a vertical tube illuminated obliquely (Table IV). Like *A. tonsa*, these animals apparently have different responses to direct and to diffuse light. Most of them show an upward movement in the dark.

The diversity of these animals, ranging from protozoans to arthropods and echinoderms, suggests that a principle of general importance is involved. A

TABLE III

Summary of light measurements

$$\text{Diffusion ratio} = \frac{\text{sum of other intensities}}{\text{highest intensity}}$$

Complete concentration = 0; complete diffusion = 15

		Diffusion ratio
Measurements in cylinder * six inches below surface		
Light position	1	1.08
	2	0.48
	3	0.15
	4	0.012
	5	0
Measurements in sea (Whitney, 1941) 5 met. below surface		
Clear sky, plane of sun perpendicular to sun		2.6
		3.2
Diffuse sky, any plane		2.0
Measurements in air 3 feet above ground		
Clear sky, plane of sun perpendicular to sun		0.13
		9.2
Diffuse sky, plane of sun perpendicular to sun		6.2
		6.8

* As the hood limiting incident light to 22.5° made the photocell insensitive to less than 20 F. C., these values are probably somewhat lower than the true ones.

TABLE IV

Summary of geotropism experiments

Animal	Phototropism in horizontal tube	Geotropism in vertical tube		Author
		In light	In dark	
Corethra plumicornis larva	+	+	−	Harper, 1907
Cyclops albidus	−	+	−	Esterly, 1907
Branchipus serratus	+	+	−	McGinnis, 1911
Daphnia pulex	+	+	−	Dice, 1914
Sagitta bipunctata	+	+	−	Esterly, 1919
Diadema setosum larva; *Paramecium*	Indif.	+	−	Fox, 1925
Holopedium gibberum	+	+	Indif.	Kikuchi, 1938
Hemimysis lamornae	+ or −	+	−	Foxon, 1940
Acartia tonsa	+	+	−	Schallek, 1942

The nauplii of *Balanus perforatus* sink to the bottom of the aquarium when taken from the dark into horizontal light, but swim up when the lamp is moved overhead (Ewald, 1912). Indif. means indifferent.

possible explanation of this effect is provided by Clark (1933). The beetle *Dineutes* moves toward the lamp if placed in a direct beam of light. If a piece of white cardboard is held perpendicular to the beam 300 cm. behind the animal, occasional circus movements appear. These become more frequent as the cardboard is brought closer to the animal, until at 10 cm. they become continuous.

This effect is attributed to the stimulation of additional ommatidia by the light reflected from the cardboard. In concentrated light, the photoreceptor will be stimulated from the front only, and the animal will then react in a typically phototropic fashion. In diffuse light, however, the photoreceptor will be stimulated from both front and side, and a different behavior appears. In the case of *A. tonsa* this results in cessation of activity, since the animal may be observed to sink passively in diffuse light.

Laboratory studies of the light reactions of animals have largely been concerned with phototropic behavior in a direct beam of light. Measurement of the light distribution in the sea shows that it is much more diffuse than in such experimental conditions. The reaction of *A. tonsa* to diffuse light in the laboratory accords with its downward movement in the ocean during the day. Its reaction in the direct light in which experiments on phototropism are usually conducted has no bearing on its behavior in nature.

Such relations may not be confined to this particular copepod. Several reports have been quoted suggesting similar behavior in other aquatic forms. The measurements of light distribution in air show that it is generally diffuse. Perhaps this will solve the riddle of why positively phototropic insects do not fly up to the sun: they may move toward a direct light but behave differently in diffuse light. Phototropism experiments in a direct beam of light need not necessarily apply to the behavior of organisms in nature.

Summary

When the copepod *Acartia tonsa* is placed in a tall glass cylinder illuminated from above, the animal swims upward. When the cylinder is illuminated obliquely, the animal sinks downward.

Measurement of the light distribution inside the cylinder shows that the behavior of *A. tonsa* depends upon the angular distribution of the light. In highly directional illumination, the animal reacts phototropically, and swims toward the light. In less directional (diffuse) illumination, the animal stops swimming and sinks passively downward.

Measurement of the light distribution in the air and in the sea shows that it is generally more diffuse than the conditions in the cylinder. The reaction of *A. tonsa* to diffuse light in the laboratory accords with its downward movement in the ocean during the day. Its reaction in the direct light in which phototropism experiments are usually performed has no bearing on its behavior in nature.

Acknowledgments

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STUDIES ON THE LIFE HISTORY OF THE MARINE ANNELID *NEREIS VEXILLOSA*

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INTRODUCTION

Nereis vexillosa Grube is the most abundant member of the larger annelids of the Pacific northwest fauna. In Puget Sound it attains a size of about 30 cm. but, as will be explained later, some heteronereid individuals may be only about 6 cm. long.

It is characteristically a cold water form occurring intertidally and in shallow water from eastern Siberia, Alaska and southward along the northwest American coast to Santa Barbara, California. Some reports extend its range as far south as San Diego. In the southern range, however, it may have been confused with *Nereis mediator* Chamberlain (Chamberlain, 1919) or with *Neanthes succinea* (Frey and Leuckart) according to Hartman (personal communication). The apparent absence of the specific egg masses, described below, from the southern range lends support to the belief that *N. vexillosa* may not occur in these waters.

The tiny eggs produced by this animal are about 0.2 mm. in diameter; they are spawned in firm, irregular gelatinoid masses, somewhat translucent, and of blue-green, greenish or brownish tints (Figure 1). These colors are most noticeable in the freshly laid eggs owing to their greater compactness prior to absorption of water by the capsular material. The firmness of the masses enables them to withstand a good deal of handling or washing about on the beach by waves without disintegrating. Hence they are often found in good condition on tidal flats where bits of seaweed or other debris collects at the waters edge. They appear never to be found in more than moderate quantities despite the abundance of the species producing them.

In so far as known, there is no other nereid worm that deposits its eggs in this manner. Commonly the eggs of other species of the genus, or of closely related genera are deposited separately in the water or are only lightly agglutinated. The size of masses varies considerably being about one to three inches in diameter, apparently depending upon the size of the female depositing them.

The identity of the egg masses has long been a puzzle to biologists and others alike, and, indeed, the present study was initiated in 1927 at Friday Harbor, Washington, primarily for the purpose of identification. In the course of the investigation, however, important aspects pertaining to the life history of the species have come to light.

It is a pleasure to acknowledge the generous support given this investigation by Director T. G. Thompson, Professor T. Kincaid, and other members of the staff of the University of Washington Oceanographic Laboratories where facilities

were provided for much of the work. My thanks are also due to Dr. Olga Hartman of the Allen Hancock Foundation for identification of the heteronereis worm and for helpful suggestions.

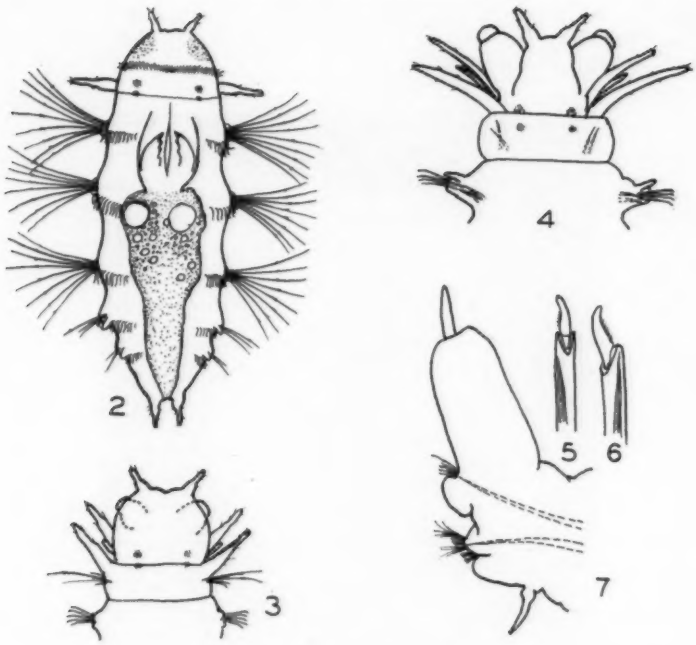
FIGURE 1. *Nereis vexillosa*. A small egg mass, about natural size.

CULTURE EXPERIMENTS AND DEVELOPMENT

The larvae resulting from culture of eggs collected in 1927 failed to survive long beyond hatching and therefore served to establish only that they were annelids of the nereid type. The failure of these to survive may be due in part to inadequate food since they were fed only diatoms and algae. This diet brought good results with *Platynereis agasszi* which was being reared at the same time (see Guberlet, 1933), but subsequent experiments have indicated that *Nereis vexillosa* thrives best in later life when animal food is provided.

In the summer of 1941 an opportunity was again presented to set up cultures at Friday Harbor. Two egg masses were found in False Bay and rearing of the larvae began on June 28. Some of the larvae were already hatching in the three to five segmented stage (Figure 2). The larvae and young worms grew slowly and were able to survive for about a week after hatching on the yolk content which became concentrated in the digestive tract. Later they were fed sessile diatoms (*Navicula sp.* and *Biddulphia laevis*) and powdered dry scallop muscle.

The rate of growth varied so greatly that on August 23 the most advanced worms, now about 57 days old, had acquired 35 segments and were about 9 mm. long, whereas the least advanced had but 15 segments and were about 5 mm. long.



FIGURES 2–7. *Nereis vexillosa.*

FIGURE 2. Five-segmented larva.
FIGURE 3. Head of eight-segmented larva to show development of peristomial tentacles.
FIGURE 4. Head of fifteen-segmented larva to show further progress of head development.
FIGURES 5 and 6. Homogomph notoseta and heterogomph neuroseta from parapodium shown in Figure 7.
FIGURE 7. Parapodium from posterior portion of body of eleven month old worm.
(Camera lucida drawings, Figures 2, 3, 4 same scale; Figures 5, 6, 7 enlarged scale.)

It was, however, still not possible to establish identity of the worms from the most advanced specimens, therefore, at the end of the summer session the animals were moved alive to the Scripps Institution of Oceanography at La Jolla, California. This was accomplished by placing ten specimens of various sizes in each of four vials with sea water and containing a wad of cotton among the strands of which the young worms found protection against violent battering in the vials while in transit. The vials were kept cool by being wrapped in wet, frequently-changed paper towels.

Upon arrival at La Jolla the specimens were changed gradually to local sea water (salinity about 3‰ above that at Friday Harbor) in small culture dishes and it was found that 25 had survived the journey. Following some additional mortality in the first month, 16 of various sizes lived for several months.

After setting up the cultures at La Jolla, about three-fourths of the water was changed in each dish every two or three days and the animals were fed dried

powdered pecten and sea mussel for a few weeks, but growth was so slow that it was decided to try feeding finely chopped pieces of freshly killed *Thoracophelia mucronata*, a polychaete worm found abundantly at La Jolla. This fresh food was given in small quantities every second or third day. With it there was a prompt response by more rapid growth. Later bits of fresh mussel were also fed but the minced worms seemed to be preferred. Not only the killed and minced worms were eaten but living specimens half the size of the young Nereis were eaten. This rapacious habit was evident even in worms only 6 or 8 mm. long that were seen to grasp and completely swallow in a few seconds others of their kind that were fully half their own size. Diatoms were also added to the dishes and these were readily eaten. The animals rejected partially decomposed food and since the object of the experiment was to keep them growing as long as possible no further experimenting with food was deemed advisable. Results indicate that *N. vexillosa* is more or less omnivorous, utilizing mostly animal food but that it is not a scavenger by preference. According to Copeland and Wieman (1924) *Nereis virens* is also omnivorous though Gross (1921) found only evidence of plant feeding.

No membranous tubes were constructed as in *Platynereis agasszi*, only flimsy tubes of sand and debris were formed with the aid of secreted mucus, and after the animals had reached a length of about 15 mm. they rarely left their tubes completely to gather food. To facilitate study, pieces of glass tubing were provided and these were readily accepted in most cases. Some animals refused to accept new glass tubes that were provided to replace those outgrown and deserted. In these instances the lack of security resulted in restlessness and failure to feed normally.

The older specimens were kept in running (uncooled) sea water pumped in from the sea. At the middle of July this water had reached a temperature of 20° C., which is about 6 to 8 degrees higher than might be expected in the natural habits of *N. vexillosa* near Friday Harbor during the summer. This may well have been a contributing factor in the failure of the worms to survive with the advance of summer at La Jolla.

In Table I is given a summary of the condition of the worms that survived ten or more months. Two of these were found dead (May 18 and May 29). The others were killed and preserved only after they had deserted their tubes and it seemed obvious that they would not survive much longer.

At the age of four and one-half months, when about 60 segments had been formed, the worms reared from the 1941 egg masses had developed specific characteristics of *N. vexillosa*. Especially characteristic are the elongate strap-like dorsal ligules (Figure 7) of the parapodia of the posterior region of the body, but the head structures and the setae of the posterior region are also distinctive (Figures 5 and 6) in older specimens.

The process of cephalization is shown in Figures 2, 3, and 4. In this development, the first setigerous segment of the early larva becomes modified to form the peristomium. The anterior dorsal pair of peristomial tentacles appear first, followed by the posterior dorsal pair which develop from the first larval parapodium. A ventral pair of anterior peristomial tentacles then appear and finally the posterior ventral pair of tentacles are in evidence when the worm has acquired about 18 to 20 segments.

TABLE I

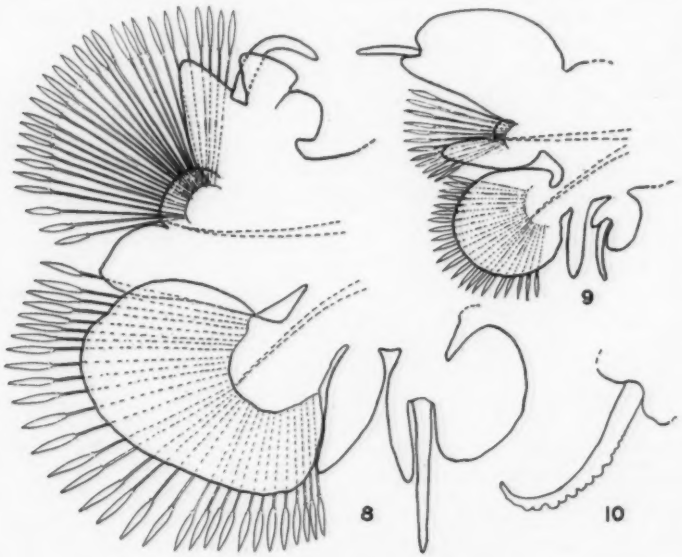
Nereis vexillosa. The seven oldest specimens from cultures set up at Friday Harbor, June 28, 1941

Date killed or found dead 1942	Number of segments	Approximate length when moderately relaxed
April 25	128	8 cm.
May 18	109	5 cm.
May 26	109	6.7 cm.
May 26	107	4.5 cm.
May 29	119	8.5 cm.
June 10	105	7 cm.
July 28	125	10 cm.

None of the specimens showed any indication of entering the heteronereis phase. In this respect they differ from *Platynereis agasszi*, several of which were found to enter this phase near the end of the first year.

SPAWNING HABITS

While culturing the larvae at Friday Harbor in 1941 a watch was kept for spawning adults in the bay. Finally from isolated small, spawning heternereids there was obtained several masses of eggs. These spawners proved to be *Nereis*



FIGURES 8–10. *Nereis vexillosa.*

FIGURE 8. Heteronereized female parapodium, middle portion of posterior body.
FIGURE 9. Heteronereized female parapodium, fourth from last segment.
FIGURE 10. Dorsal cirrus, heteronereized male parapodium.
(Figures 8, 9, 10 camera lucida drawings to same scale.)

vexillosa Grube thus confirming the identification of the worms reared experimentally from egg masses collected in the field. Heteronereized parapodia are shown in Figures 8 and 9. Heteronereis males have 25 parapodial segments in the anterior portion of the body while the females have 27 such segments (count of 12 males and four females). In the males the dorsal cirrus of the heteronereized segments bears a series of wart-like protuberances on the ventral surface (Figure 10) while in the females examined these cirri were smooth.

The spawning worms were obtained only at night while collecting with a light at the end of the pier in front of the laboratories. They appeared only in small numbers usually an hour or two before midnight and were mingled with spawning swarms of the smaller species *Platynereis* (formerly *Nereis*) *agassizi* which on all occasions was the first of the two to appear swimming at the surface. It was not possible with these few observations to establish any correlation of spawning with phases of the tide or moon as has been done with other marine worms (cf. Woodworth, 1907; Lillie and Just, 1913; Guberlet, 1933).

Only small individuals of *N. vexillosa* were seen spawning and from what has subsequently been learned through the above rearing experiments, it seems certain that these were all spawning for the first time at the age of one year. Not only were some of the experimentally reared worms as large at the age of 10 to 11 months as some of the spawning worms but there was also indication of approach to sexual maturity. One of the worms reared at La Jolla was killed April 25, 1942 after having deserted its tube. Upon examination it was found to contain many eggs which were very small and not yet ripe, but their large numbers might be interpreted to indicate that spawning would normally have occurred in the coming summer. A second worm that died May 29 also showed many eggs. However, it seems certain that in nature some heteronereis individuals must be older than one year at spawning since egg masses of much greater size than those known to have been spawned by the smaller specimens have been found. This is more fully discussed later.

In nature more males than females were observed swimming at the surface. They are the first to come to the nuptial party where, as scattered individuals, they suddenly appear from below and rise to the immediate surface, swimming a few moments there in spirals and loops and then disappearing into the deeper water or the darkness beyond the range of the collector's light. They continue unabated in numbers and vigor as the females a little later appear to join the dance, a dance which seemingly is a climax that marks the ends of their lives, for none of the exhausted individuals kept in captivity was observed to live more than a few days following the act of spawning.

Isolated small heteronereis females ripe with eggs were induced to spawn almost instantly when a few drops of sperm laden water were added to the water in which they were isolated. This is similar to the findings of Lillie and Just (1913) for *Nereis limbata* and of Just (1929) for *Platynereis dumerilii*. In large battery jars the act of spawning by the female *Nereis vexillosa* consists of coming to or near the surface and suddenly exuding a mass of eggs which instantly agglutinates (Figure 11). She then passively sinks to the bottom together with the mass. A few moments later she frees herself from the mass which remains demersal and which in a few hours swells to about three or four times its original size through the absorption of water. The spawning of these demersal masses

in water just beyond the low tide limit may account for the finding of fewer egg masses than are commensurate with the number of worms.

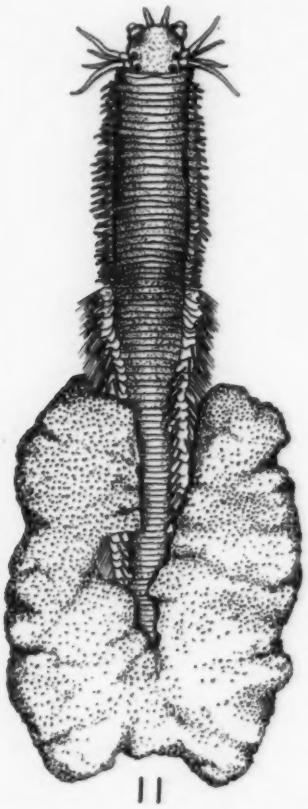


FIGURE 11. *Nereis vexillosa*. Method of egg deposition by small heteronereis. (Free hand drawing.)

DISCUSSION

An answer has been found as to the identity of the egg masses, and certain features of the life history of the species have been discerned. In the process of interpreting the observations, however, another biologically important question arises, namely, does *Nereis vexillosa* possess a diversified life history in which there may be recognized several types of reproductive individuals, as indicated for *Platynereis dumerilii* by Hempelmann? In the latter species at Naples, Hempelmann (1911) distinguished (1) small sexually mature nereis (i.e., atokus) individuals that gave rise to heavily yolked eggs producing characteristic larvae which he called "nereidogene"; (2) small heteronereis forms producing less

heavily yolked eggs developing into pelagic larvae called "planktogene"; (3) large heteronereis forms with eggs as in the small form but whose larvae have not been investigated. He found that after spawning, the small nereis form may in experimental cultures be transformed into a small heteronereis form and produce young for the second time and is therefore dissogenous. The nereis form may also grow to a relatively large size and then transform to the large heteronereis but the steps involved in arrival at the large form in this phase are uncertain. It is believed that entrance into the heteronereis form and its spawning marks the end of life for the individual.

More recently Just (1929) also worked on the Naples species and in so far as his investigation was carried, the findings of Hempelmann were verified.

It has been noted by H. P. Johnson (1901) that the heteronereis form of *Nereis vexillosa* occurs in individuals of 56 mm. and upward in length but the maximum length is not given. The same author reports that sexual maturity is frequently arrived at by the species without it becoming heteronereized. Ricketts and Calvin (1939) report finding many large heteronereis of *N. vexillosa* but the authors never found these to be free-swimming. The supposition is, however, that they do spawn, and indeed the finding of egg masses in Puget Sound that are much larger than those known to have been spawned by small heteronereis forms of the species substantiates this.

What the destiny of the worms reared from the Friday Harbor material would have been normally can be only a matter of conjecture. In this connection it may be significant to note that the number of segments in several large (14 to 21 cm. long) *N. vexillosa* collected in the field at Friday Harbor was 142 to 152 in the nereis phase, whereas the spawning heteronereids taken in the same region had only 63 to 96 segments. The latter figure is less than the number occurring in the seven most advanced specimens reared in cultures (Table I). These specimens had 107 to 128 segments and the length of some was greater than the spawning worms. This may mean that the reared worms were destined to reach sexual maturity only in a more advanced nereis phase or in a large heteronereis phase. Much additional study is needed to answer this question. The great range in size that is possible in the heteronereis of *N. vexillosa* should make it an ideal species for such a study if culture problems can be overcome.

Summary

Nereis vexillosa deposits its eggs in firm irregular gelatinoid masses which vary in size from about one to three inches in diameter.

Spawning of small (6 to 8 cm. long) heteronereids of this species was observed to take place an hour or two before midnight. The eggs which are demersal are apparently spawned in water at or just beyond the extreme low tide level and this habit may account for the finding of fewer egg masses than seems commensurate with the number of the species producing them. Isolated heteronereis females were induced to discharge their eggs by the introduction of spermatozoa into the water.

Worms cultured in the laboratory from egg masses collected on the beach throve best on fresh animal food. A number of young worms transported from Friday Harbor to La Jolla attained a maximum length of 10 cm. at the age of 13 months after hatching. All had acquired specific characteristics but none

became heteronereized. The transformations taking place during cephalization are similar to that occurring in other nereid worms.

Since the above maximum size of the reared worms is comparable to that of the small spawning heteronereis forms, it appears that the latter were spawning at the age of about one year. Much larger nereis and also heteronereis individuals are known to occur in this species but the time and steps involved in their development are unknown.

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THE REPRODUCTIVE PROCESSES OF THE FISH, ORYZIAS LATIPES

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Introduction

The study of experimental vertebrate embryology has been limited to the breeding seasons of the lower forms with the exception of the amphibia (Rugh, 1941) where ovulation can be induced at almost any time of the year. A study of the reproductive processes of the fish *Oryzias latipes*, the Japanese medaka, was planned in the hope that its eggs might also be made available for more investigations in the experimental field.

Materials and Method

The fish in a sexually mature condition were obtained from a West Coast importer. They were kept in ten-gallon aquaria in a south window, at room temperature and in spring water which was oxygenated by Nitella. The food consisted of freshly collected Tubifex which were cleaned in running water, Daphnia, and some dried fish food. Artifical lighting was supplied only under experimental conditions. This consisted of a 75-watt bulb held above each tank, the light being increased with a metal reflector. When light was to be eliminated this was accomplished by covering the entire tank with a light-tight cardboard box which allowed sufficient circulation of room air and maintenance at room temperature.

The female urino-genital system was studied from cleared whole mounts and serial sections, as well as in the living condition. For a study of the ovulation process females were anesthetized at the appropriate time in MS 222, one part to 3000 of spring water, pinned in a permoplast dish and dissected.

Observations and Experimental Data

The female reproductive system.

The structure of the reproductive tract of the female *Oryzias latipes* (Figures 1–8) was studied as preliminary to observations and experiments on the sexual cycle. The ovary and the oviduct are essentially like those previously described for other poeciliids. The ovary is a median, unpaired sac-like organ (Figures 7 and 8) filling most of the body cavity behind the posterior edges of the liver. It is composed of a large number of follicles in different stages of development, all facing a central lumen which is somewhat occluded by the projecting ripe follicles. These follicles are attached to the thin, muscular ovarian wall on all

[1] Submitted in partial fulfillment of the requirements for the degree of Master of Science at New York University.

sides except the dorsal. Through this dorsal wall the lumen and the follicles are visible even in the intact ovary.

The developing oocytes are practically identical with those of *Fundulus heteroclitus* (Marza, Marza, and Guthrie, 1937; Solberg, 1938), except with respect to size. A small oocyte with a large germinal vesicle becomes transformed into a large egg containing a mass of yolk and oil drops surrounded by a thin layer of cytoplasm. During this growth phase of maturation a thick chorion with long fibers attached to its external surface is laid down around the ovum by the follicle cells. This is a protective outer, non-living membrane comparable to the jelly capsules of the amphibia.

The oviduct is a single thick-walled straight and non-glandular tube about one millimeter in length (Figures 3–8), unpaired as in *Xiphophorus helleri* (Essenberg, 1923; Bailey, 1933; Regnier, 1938) in which species it is not the homologue of the Mullerian duct (Essenberg, 1933). The walls consist largely of circular muscle fibers (Figure 5). The lumen of the oviduct is lined with epithelium which is columnar near the ovary and becomes squamous toward the external opening. Numerous folds in the epithelium of the contracted oviduct allow considerable expansion as the eggs pass rapidly through. There are no glands present, as there are in the amphibian oviduct. The thick muscular walls of the ovary and of the oviduct are histologically continuous, and show no appreciable difference except in regard to total thickness. The duct extends from the posterior end of the ovary to open behind the urino-genital papilla, between the anus and the mesonephric duct (Figure 8). The mesonephric duct descends in the muscle of the body wall posterior to the body cavity to run parallel to the oviduct. Before it does this it bends anteriorly almost to the body cavity and then sharply posteriorly (Figure 8). In the region near the external opening there is no muscular wall, but one is soon organized from the surrounding tissue. In the region of the two loops the lumen is large, the muscular wall is relatively thin, and there are many villi in the mucosa.

The urino-genital papillae are a pair of protuberances from the ventral surface of the female between the anus and oviduct opening (Figure 8). They extend posteriorly and ventrally from their attachment to the body wall, covering the opening of the oviduct, and are grown together in the mid-line for most of their length. The papillae have a thick cortex of stratified epithelium and a highly vascularized medulla.

PLATE I

FIGURE 1. Stained section through external opening of mesonephric duct.
FIGURE 2. Stained section anterior to Figure 1, through mesonephric duct.
FIGURE 3. Stained section anterior to Figure 2, level of mesonephric duct loop.
FIGURE 4. Stained section anterior to Figure 3.
FIGURE 5. Stained section anterior to Figure 4.
FIGURE 6. Stained section through posterior end of body cavity.
FIGURE 7. Stained section through posterior part of ovary.
FIGURE 8. Drawn from a cleared whole mount.

B—body wall; C—connective tissue; D—mesonephric duct; E—edge of rupture; F—fat; I—intestine; L—loop of mesonephric duct; M—muscle; N—capillaries on follicle; O—oviduct; P—body cavity; R—fin ray; S—scale; T—threads on egg; U—urino-genital papilla; V—ovary; X—overgrowth of body wall epithelium; Y—follicle wall; Z—constriction.

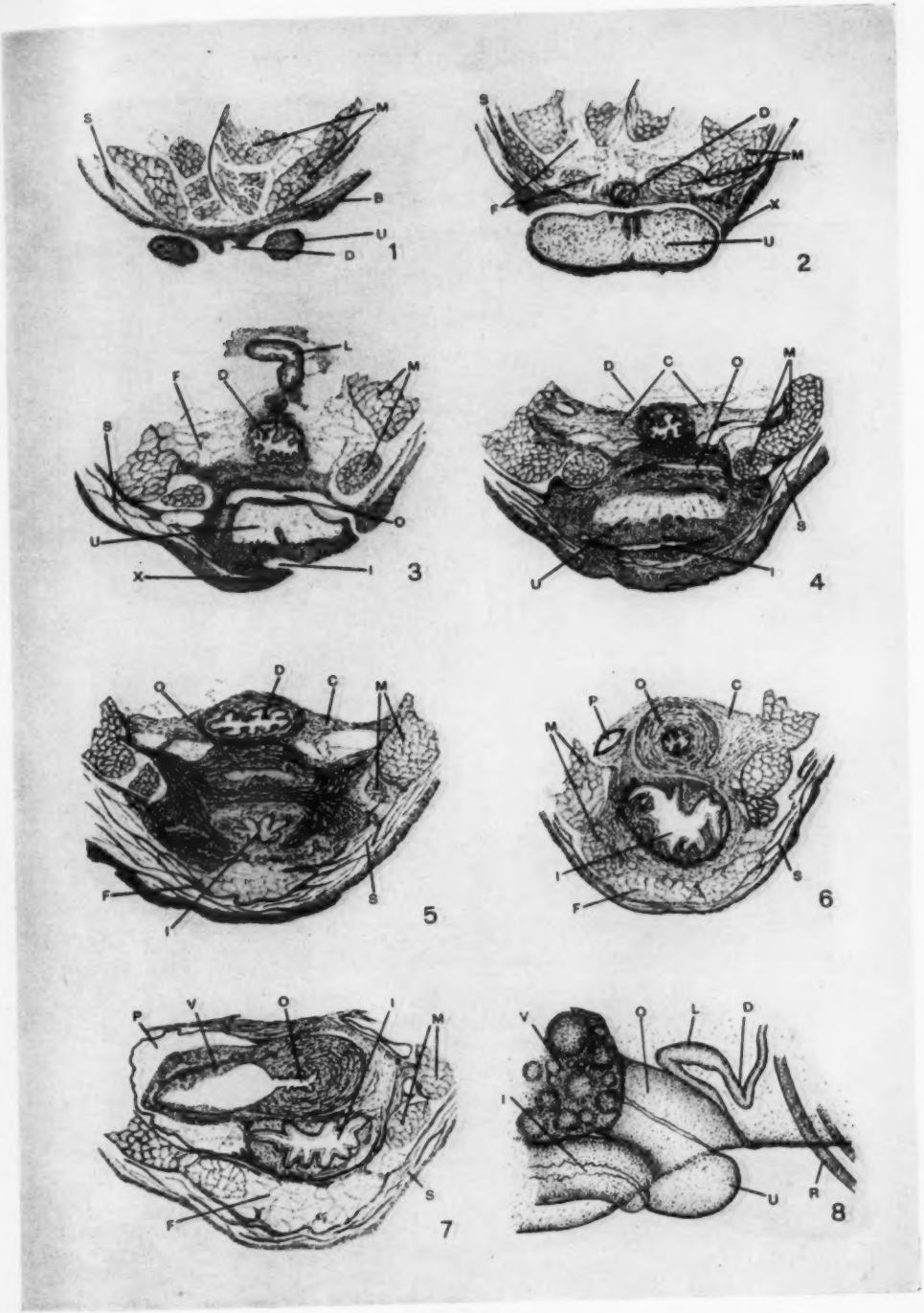
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PLATE I

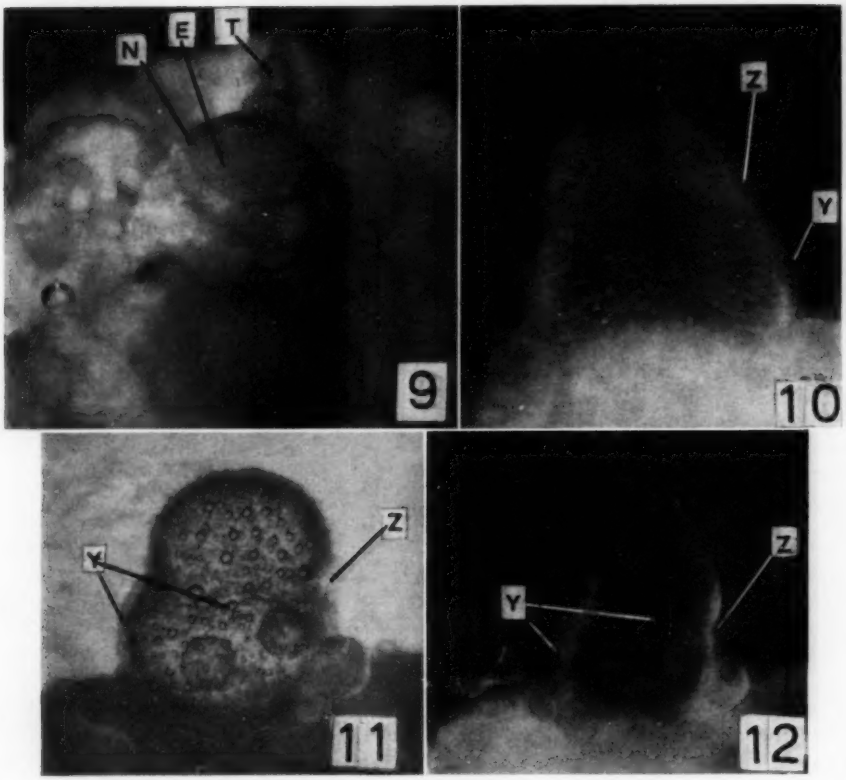

PLATE II

FIGURE 9. Fixed ovary with the dorsal wall removed showing rupture and emergence of egg.
FIGURE 10. Profile view of living egg one-third emerged from follicle.
FIGURE 11. The same egg half emerged, transmitted light.
FIGURE 12. The same, reflected light.

Ovulation and egg transport.

The ovulation process was observed in more than twenty ripe ovaries, either removed from the fish at the appropriate time or observed in partially dissected fish under anesthesia (Figures 9–12). Some of the observations were made directly through the dorsal wall of the intact ovaries, others were made after removal of this dorsal ovarian wall.

The rupture of the ripe follicle appears in the center of a vascular plexus on the side toward the lumen (Figure 9). The hole is generally oval in shape at the beginning of the process, and as it increases in size the blood vessels around it are stretched but do not rupture. As the egg protrudes (Figure 10), the packed threads attached to the chorion become unwound, and the edges of the hole constrict the egg appreciably (Figures 11–12). There is no noticeable increase in the rate of emergence after the egg is half extruded, probably because intraovarian pressure from the muscular walls prevents any sudden eruption. The process of rupture and emergence of the egg from its follicle is very similar to that described for the frog (Rugh, 1935) except that in the frog the egg is released into the body cavity. At the end of the process, from 20 to 30 distorted eggs fill the lumen of the ovary.

The exact duration of the normal process of ovulation cannot be stated since the anesthetized and partially dissected fish are probably not physiologically normal. However, it is possible to estimate approximate times. During these observations most of the eggs in intact ovaries took from 15 to 45 minutes to be extruded. The longer intervals were probably due to the ovary becoming moribund. If intraovarian pressure is removed by tearing the dorsal wall of the ovary, as was done frequently to obtain a better view of individual eggs, an egg may emerge in ten minutes from the time of the first visible rupture. Examining fish taken from an aquarium at intervals during the period of ovulation, it was found that under normal conditions all the fish ovulate completely during a period of about an hour and a half, thus setting that interval as an absolute maximum. Since all fish do not begin to ovulate simultaneously, this estimate is too high for any single specimen and it is presumed that an individual egg normally takes about ten to 20 minutes to emerge from the follicle, and that a single fish ovulates all of its eggs in less than one hour.

The forces causing the initial rupture of the follicle and the extrusion of the egg are unknown. At no time were movements of the ovarian wall or of individual follicles noticeable, although the presence of smooth muscle fibers in both implies the possibility of such movements during ovulation. Furthermore, the whole ovary often undulates remarkably when a fish is first dissected, the contortions resembling euglenoid movement. It is interesting that these movements did not cause rupture of the follicles. Until the forces causing ovulation are known they may be presumed to be similar to those which act in other poikilothermous forms (Rugh, 1935).

The eggs are probably forced into the oviduct by muscular movement of the ovary similar to those observed under the stimulation of dissection. The muscular oviduct and the muscles of the adjacent body wall may also aid in moving the eggs quickly through the short oviduct.

Fertilization.

The mature eggs are not stored in the ovary for more than a few hours, for it was found that normally they are laid almost immediately after ovulation, provided a sufficient number of males is present to supply the incentive. The eggs are laid all at once by the female and are fertilized immediately, while they remain attached to the female by chorionic threads. Egg-laying was not observed, it being a very rapid process, but most of the courtship and fertilization process was observed a number of times. Each time it lasted less than 60 seconds. Kamito (1928) stated that the entire process takes about 35 seconds, and the present observations tend to confirm that statement. After fertilization the eggs are accidentally brushed off on plants, during the day. They remain attached to vegetation during early development.

The maturation cycle.

The maturation cycle of the medaka is 24 hours long and is very regular since the eggs are almost invariably laid in the early morning, although Kamito (1928) reported they may also be laid in the evening. Observation of more than 150 females over a period of three months showed only one possible exception to the rule that the eggs are laid in the early morning. About 75 per cent of all of the adult female fish in the aquarium produce eggs daily. The eggs are laid almost immediately after ovulation. Dissection of numbers of stock fish taken at random at different times of the day never showed ovulated eggs in the ovary before about 1 A.M. or after about 9 A.M. On two occasions six and eight fish were dissected about an hour and a half before the usual time of egg laying and were found to be ovulating or about to ovulate. Thus the time at which the eggs are laid indicates the approximate time of day they were ovulated.

One other important fact was discovered by daily observation of medakas in stock aquaria, although not under experimental conditions. These fish were in aquaria artificially instead of naturally aerated, but otherwise under the same conditions as the other stock and experimental fish. There was no photoperiodicity in these aquaria since lights were above them constantly. These fish did not show the regularity of ovulation that characterizes the species. Newly-laid eggs could be found during any period of the day, and the fish in an aquarium did not lay eggs at approximately the same time, even though the conditions for all were identical.

Experimental Data

The knowledge that the eggs are normally ovulated and laid near dawn, and that unusual lighting conditions upset the regular cycle, suggested that diurnal light changes might be important in controlling the normal cycle. To test this hypothesis experiments were performed as follows:

In the first, eight male and six female specimens of *Oryzias latipes* were put in each of two five-gallon aquaria with food, water, and flora from stock aquaria. One aquarium was covered with a light-tight box from 8:30 P.M. until 8:30 A.M. (Tank I) and the other was covered from 8:30 A.M. until 8:30 P.M. (Tank II).

For the remaining 12 hours each tank was lighted by a 75-watt bulb held directly above in a metal reflector. After one week the lighting schedule for each tank was reversed. The temperature varied with that of the room. The time the eggs were laid was determined either by frequent observation or by noting the stage of cleavage when the eggs were first seen and removed from the female. The developmental rate of the Oryzias egg has been worked out for laboratory temperatures (Rugh, 1941). The first division occurs over an hour after fertilization, and the next two divisions at 45 minute intervals. Results are summarized below (Table I).

TABLE I

Date	Tank I (covered all night)	Tank II (covered all day)
Feb. 2	No eggs all day.	No eggs all day.
3	No eggs all day.	No eggs all day.
4	One laid eggs between 8:30 and 9:30 A.M. No others laid eggs.	One laid eggs between 8:30 and 9:30 P.M. No others laid eggs.
5	One laid eggs between 8:30 and 9:30 A.M. No others laid eggs.	One laid eggs between 8:30 and 9:30 P.M. Others did not lay.
6	One laid eggs between 5 and 8:30 A.M. One laid between 8:30 and 8:40 A.M. No more laid eggs.	Two laid eggs between 5 and 8:30 P.M. One laid eggs between 8:30 and 9 P.M.
7	Two laid eggs between 6 and 8:30 A.M. One laid between 8:30 and 8:40 A.M. No more.	One laid eggs at 9 P.M. One laid eggs between 7 and 8:30 P.M. No more laid.
8	Two laid eggs between 8 and 8:30 A.M. One laid between 8:30 and 9:30 A.M. No more laid.	Tank uncovered at 7:10 P.M. No eggs up to 7:40 P.M.
9	*Schedule reversed. Both tanks uncovered until 2:30 P.M., then tank II was covered.*	
	Tank I	Tank II
10	One with eggs at 2:45 P.M. No other eggs at 8:30 P.M. or at 10 P.M.	No eggs at 8:30 A.M. One laid between 8:30 and 10:30 A.M., another between 10:30 and 11 A.M., another between 11 A.M. and 1 P.M.
11	No eggs at 8:30 A.M., or at 8:30 P.M.	No eggs up to 9 P.M.
12	One laid eggs between 7:30 and 8:20 P.M. Two laid between 8:20 and 10 P.M. No others laid eggs.	No eggs at 8:30 A.M. One laid eggs between 8:30 and 9:50 A.M. No other eggs all day.
13	No eggs at 8:30 P.M. Three laid between 8:30 and 9 P.M. No others laid.	No eggs at 8:30 A.M. One laid between 8:30 A.M. and 1 P.M. No other eggs all day.

The same type of experiment was started with a new set of fish on March 23 (Table II). Twenty females which had been laying eggs between midnight and 8 A.M. without exception for several weeks were put in a ten-gallon aquarium with 22 males, under conditions already described. The aquarium was covered from 8 A.M. until 8 P.M. and lighted with a 75-watt bulb from 8 P.M. until 8 A.M. As controls, six females from the same lot were kept in a community tank and these fish continued to lay eggs nearly every morning. The results appear in the following table:

TABLE II

Date	Observations at 8 P.M.
March 23	No eggs; none laid all day.
24	No eggs; none laid all day.
25	No eggs; none laid all day.
26	Fifteen with eggs, mostly in the two-cell stage, some up to eight cells—i.e., one to three hours old. None laid the rest of the day.
27	Ten with eggs, mostly in the two-cell stage, some up to eight. None laid the rest of the day.
28	Eleven with eggs, mostly in the two-cell stage, some up to eight. None laid the rest of the day.
29	Thirteen with eggs, mostly in the two-cell stage, some up to eight. None laid the rest of the day.
30	Tank covered at 10:30 A.M. and kept covered until 8 P.M. March 31st. Not examined for eggs at 8 P.M.
31	One with newly-laid eggs at 8:30 A.M. Three with eggs in late stages of cleavage at 8 P.M., laid since 8:30 A.M.
April 1	No eggs at 8:40 A.M., nor at 8:40 P.M. Eleven with eggs at 9 P.M. None laid later.

At the end of the experiment the fish were returned to a normal daylight schedule and within two days as many as 75 per cent of the females were laying eggs before 8 A.M. each day.

DISCUSSION

These experiments indicate that the ovulatory cycle of *Oryzias latipes* is in some way correlated with diurnal photoperiodicity. With but four isolated exceptions among the 32 experimental females, reversal of the photoperiodicity changed the time of ovulation, as indicated by egg-laying, by approximately 12 hours. In the first experiment there was an appreciable change in addition to the reversal during the experiment, as the fish had been laying eggs near the middle of the day. In the second experiment two reversals occurred, including the return to normal conditions. The two or three day lag between reversal of the photoperiodicity and appearance of the first eggs is assumed to be caused by an adjustment of the fish to the new lighting conditions.

The exceptional results are easily explained. The two fish in tank II on February 10 undoubtedly were not yet adjusted to the sudden change in photoperiodicity; the one in tank II on February 13 was in poor condition, having been taken from slightly foul water. The exceptional fish on March 31 was the result of the upset of photoperiodicity on the previous day.

These facts suggest the manner in which photoperiodicity may regulate the ovulatory cycle in *Oryzias latipes*. The species is physiologically and genetically adjusted to a daily cycle. This means that 20 to 30 relatively large eggs must be matured, ovulated, and laid in the space of 24 hours, the bulk of this time being utilized in the maturation process. Ovulation (i.e., rupture and emergence of the egg from the ovary) must normally occur approximately at dawn because these studies show that oviposition follows shortly thereafter and *Oryzias latipes* has long been known to produce its eggs early in the morning. It is inconceivable that the temperature of a 15-gallon tank of water would change as abruptly as does the light factor early in the morning. Of the two variables, it seems most

likely that since the temperature is relatively constant and the light factor is extremely variable, that this latter factor is the trigger which sets off the ovulation and oviposition reactions. Light in itself may not be necessary in the sense that it acts through the sense organs to bring about breeding reactions for these fish may ovulate on occasion and deposit their eggs even in total darkness, although such reactions are not predictable. It is the thesis of this paper that the stimulus of light brings about an increase in the metabolic activity of the fish and that this activity in turn sets off the breeding reactions which have otherwise been rather quiescent. By direct observation it can be shown that the periods of light and dark do regulate the physical activity of the fish and those which have been in darkness for a considerable period fail to respond normally to tactile stimuli and generally swim very sluggishly. This has been previously reported by Spencer (1929) who found the sunfish to be quiescent at night, and Shaw, Escobar, and Baldwin (1938) who found the locomotor activity of goldfish to be much reduced in reduced sunlight. The medaka is a very active fish during the day so there may be a considerable difference in the amount of energy available for the maturation process between periods of rest (in darkness) and of activity (in light). There is no suggestion that radiant energy is thereby transformed into metabolic energy but rather that light which penetrates the water stimulates the fish to activity which activity in turn utilizes stored metabolic energy. This energy might otherwise be utilized in the maturation process.

Such a thesis does not preclude the possibility of maturation (and ovulation) in fish which are continuously active but that the reproductive cycle of such fish should be longer and more irregular. These observations confirm such a proposition. But under the normal ration of light and darkness the fish will ovulate near the end of the period of darkness no matter what time of day this happens to be. Further confirmation of this thesis might be obtained by enucleation of the females prior to a series of observations, but we would have to go further and attempt to control the chromatophores as well. This would be extremely difficult.

Other species studied have longer cycles, so no direct comparison is possible. It is not impossible that light may also affect these longer cycles, particularly if it can be proven that certain parts of the spectrum are more beneficial than others, such as ultra-violet or the other extreme, infra-red. But this suggested relationship is not offered as the explanation of the phenomena in Oryzias since it produces eggs at almost all seasons. Barney and Anson (1921) and Turner (1936, 1937, 1938) noted a direct relation between seasons and the reproductive cycles in several poeciliids. But these authors associated the breeding reactions more with seasonal temperature than light changes. Dildine (1936) did not agree with Turner's (1937) conclusions after studying *Lebistes reticulatus*. Craig-Bennett (1930) found no effect of variation of light on the cycle of *Gasterosteus aculeatus*.

Matthews (1939) and Burger (1939b), both using the male Fundulus, state that light is not essential and that it has no effect on the spermatogenetic cycle but that temperature is the all-important environmental variable. Matthews showed that low temperatures retarded spermatogenesis and Burger stated that a low light ration of one and a half hours per day kept the fish sexually inactive at 6–10° C., but that many spermatazoa were developed with the same light

ration at 14–20° C. It is logical to assume that the low temperature would retard any biological process and Burger admits that a basic light ration of one and a half hours per day is needed to keep the fish sexually active.

Although other environmental factors may be involved, under normal circumstances the time of ovulation (hence also the prior period of maturation) in *Oryzias latipes* is related to the diurnal periodicity, probably through the regulation of the states of activity during the daily cycle rather than through any intrinsic value in light (energy) itself.

Summary and Conclusions

1. The female urino-genital tract of *Oryzias latipes* is, in general, similar to that of other poeciliid fish. The ovary is an unpaired, hollow organ consisting of a thin but strong wall, lined with developing follicles on all sides except the dorsal. The development of the oocytes is similar to that in *Fundulus heteroclitus*. The oviduct is a short, muscular tube with no known function except that of egg transport.

2. The ovulation process is not cataclysmic, but takes an average of about 30 minutes under the conditions stated. Contraction of the ovary as a whole is not necessary, although this does not preclude the possibility of action of smooth muscle in the walls of the follicles themselves. The force causing the initial rupture of the follicle is unknown.

3. The eggs are laid almost immediately after ovulation, either just before or during copulation. Copulation consists of a definite series of actions by both fishes, and the sperm and eggs are shed simultaneously.

4. The fishes normally ovulate just before dawn, and inverting the periods of light and darkness as compared to natural conditions causes ovulation to be shifted from the time of the natural dawn to the time of the artificial dawn. It was suggested that light governs the time of ovulation by regulating the general metabolic activity of the female. The eggs are matured during a period of quiescence and are released at the beginning of the period of activity, stimulated by light. In the normal daily cycle light is probably the most important environmental factor, acting as a stimulant to general activity and hence to ovulation and oviposition in *Oryzias latipes*.

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